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- a) contacting a compound with a MLK protein and a substrate therefore, wherein the MLK protein is selected from the group consisting of MLK1, MLK2, MLK3, DLK, LZK, and combinations thereof;
  - b) measuring the level of MLK activity, wherein the MLK activity is selected from the group consisting of kinase activity and an ability to bind a SEK1 protein; and
  - c) comparing the level of MLK activity in the presence of the compound with the level of MLK activity in the absence of the compound, wherein a decrease in MLK activity in the presence of the compound is indicative that the compound has an ability to inhibit MLK activity.
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#### REMARKS

In the Official Action of March 26, 2001, the Examiner has stated that applicant is not entitled to the benefit of the filing date of the provisional application. The reason for this position is the allegation that the full scope of the claims is not supported by the disclosure in the provisional application. Specifically, the Examiner asserts that the claims embrace concepts which are broader than the concepts disclosed in the provisional application. Applicant respectfully disagrees with this position.

Initially, applicant points out that the standard for compliance with the requirements of 35 U.S.C. 112, first paragraph, is that the scope of the enabling disclosure is sufficient to put one skilled in the art in possession of the invention without requiring unnecessary experimentation on a trial-and error basis. *In re Bowen*, 181 USPQ 367 (CCPA 1967). Moreover, it is not necessary for the claims to be literally supported by the disclosure to satisfy the written description requirement. *In re Gardner*, 177 USPQ 396 (CCPA 1973).

Turning to the specifics of the present claims and provisional application, the invention broadly covers a method for determining whether a potential drug candidate inhibits neuronal cell death by contacting the drug candidate with neuronal cells having an activated MLK activity. The number of cells which die can be used as a measure of the ability of the compound to inhibit neuronal cell death. This can be further used as an indicator to select a drug candidate for the treatment of Huntington's Disease.

For instance, page 6 of the provisional application states that MLK activates the SEK1-JNK pathway to induce cell death in neuronal HN33 cells. In addition, page 2 states that the expression of mutated huntingtin induces apoptotic cell death in HN33 cells, while normal huntingtin, which is associated with MLK, has no toxic effect on these cells. Applicant submits that the state of the art is such that one skilled in the art, having possession of this information, could reasonably conclude that an inhibitor of MLK activity in neuronal cells would lead to a decrease in the induced apoptosis of such cells. See, for instance, the Miller et al. patent (U.S. Patent No. 6,060,247), which has been cited as prior art in the Office Action, and which discloses a generalized method for identifying inhibitors of apoptosis.

Accordingly, applicant is fully entitled to the benefit of the provisional application filing date. It is noted, however, that entitlement to the provisional application filing date does not impact the citation of prior art by the Examiner in this Office Action.

Claims 1-6, 9-11, 14-21 and 44-45 stand rejected under 35 U.S.C. 112, first paragraph, on the basis that the full scope of the claims is not supported by the specification. This ground of rejection is respectfully traversed.

The claims have now been amended to further identify the MLK kinase activity as MLK1, MLK2 and MLK3, DLK and LZK kinase activity. All five of these kinases are part of the same MLK family of kinases as shown in the relevant literature. See, for instance, S.E. Merritt et al., *The Journal of Biological Chemistry*, pages 10195-10202 (1999) and S. Hiroyuki et al., *The Journal of Biological Chemistry*, pages 28622-28629 (1997). Copies of these references are enclosed for the Examiner's convenience. These references are cited as showing that the art recognizes that the MLK family includes at least five related kinases. This is not intended, however, to exclude other, related MLK kinases which may be subsequently identified by those skilled in the art as part of the MLK family.

The mutated proteins of the present invention have also been more fully identified in the dependent claims.

Claims 7-8, 12-14, 22-23, 28 and 31 have been rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. This ground of rejection is also traversed.

Claims 7-8 and 12-13 have been amended to correct improper claims dependency, and claims 22-23 have been cancelled. Claim 14 has also been amended to provide more consistent claim language.

Claims 1-2, 6, 9, 14, 19, 21, 24, 28-31 and 45 stand rejected under 35 U.S.C. 102(e) as being anticipated by Miller et al. (U.S. Patent No. 6,060,247). This ground of rejection is respectfully traversed, and reconsideration is requested.

The Miller et al. patent relates to postmitotic neurons infected with an adenovirus vector at an elevated rate of infection. These neurons express gene products encoded by DNA molecules contained within the vector. A method for the identification of substances that inhibit apoptosis is also disclosed in the reference.

The Examiner states that Miller et al. discloses that MEKK1 induces apoptosis, and would have been known to phosphorylate SEK1. According to the Examiner, this reference is believed to meet the limitation in the claim that the activated MLK activity includes the ability to phosphorylate a SEK1 protein.

Initially, applicant notes that the present claims are not directed to inducing apoptosis in cells, or to phosphorylating SEK1. Rather, claim 1, for example, is directed to a method of identifying inhibitors of apoptosis. Although the Miller et al. patent also discloses a method for identifying inhibitors of apoptosis, the method of the reference involves infecting neurons with an adenovirus vector encoding p53. This is not the method employed in the present application.

Moreover, the amended claims do not include SEK1 phosphorylation as an activity which must (although it may) be present in the neuronal cells of this invention. It is applicant's view that any linkage between the phosphorylation of SEK1 and the inhibition of apoptosis, as suggested in the Office Action, could only have been obtained from hindsight based on the disclosure contained in the present application.

Claims 19, 21, 24 and 27-31 stand rejected under 35 U.S.C. 103(a) as obvious over any one of Tibbles et al., Rana et al. and Hirai et al., each in view of Au-Young et al. This ground of rejection is also traversed.

Claim 19 has now been amended to exclude the requirement that the ability to phosphorylate SEK1 is an activity which is required to be induced in neuronal cells. The remaining claims have been cancelled without prejudice.

In view of the foregoing facts and reasons, this application is now believed to overcome the remaining rejections, and to otherwise be in proper condition for allowance. Accordingly, withdrawal of the rejections, and favorable action on this application is solicited. The Examiner is invited to contact the undersigned at the telephone number listed below if this is believed to facilitate allowance of this application.

Respectfully submitted,

by William G. Gosz  
William G. Gosz  
Reg. No. 27,787  
Ropes & Gray  
One International Place  
Boston, MA  
Attorneys for Applicant(s)  
Tel. No. (617) 951-7000

DATE: June 27, 2001



## MARKED-UP CLAIMS

1. (Four Times Amended) A method for assessing a compound's ability to prevent neuronal cell death occurring in a mammal susceptible to or having a neurological condition, comprising:

a) contacting a compound with cultured neuronal cells having activated MLK activity, wherein activated MLK activity is selected from the group consisting of [an enzymatic activity] MLK1 activity, MLK2 activity, MLK3 activity, DLK activity, LZK activity, and an ability to bind a SEK1 protein [and an ability to phosphorylate a SEK1 protein]; and

(b) determining the number of cultured neuronal cells that die;

wherein a decreased number of dead cultured cells in the presence of the compound compared to the number of dead cultured neuronal cells in the absence of the compound is indicative of the compound's ability to prevent neuronal cell death.

2. (Twice Amended) The method of claim 1, wherein the neuronal cells are expressing a mutated protein selected from the group consisting of polyglutamine stretch-expanded huntingtin or C-terminal 100 amino acids of amyloid precursor protein, or treated with a neurotoxin to induce apoptosis.

7. (Twice Amended) The [compound of Claim 46] method of claim 1, wherein the [neurological condition is a] neuronal cell death occurs in a mammal having a neurological disease whereby glutamate or kainic acid mediated excitotoxicity is involved in neuronal cell death.

8. (Twice Amended) The [compound of Claim 46] method of claim 1, wherein the [neurological condition is a] neuronal cell death occurs in a mammal having a neurological disease comprising Huntington's disease, Parkinson's disease or Alzheimer's disease.

9. (Three Times Amended) A method for assessing a compound's ability to prevent neuronal cell death occurring in a mammal susceptible to or having a neurological condition, comprising:

a) contacting a compound with cultured neuronal cells expressing a mutated protein selected from the group consisting of polyglutamine stretch-expanded huntingtin or C-terminal

100 amino acids of amyloid precursor protein, or treated with a neurotoxin to induce neuronal cell death; and

(b) determining the number of cultured neuronal cells that die;

wherein a decreased number of dead cultured neuronal cells in the presence of the compound compared to the number of dead cultured cells in the absence of the compound is indicative of the compound's ability to prevent neuronal cell death.

12. (Twice Amended) The [compound of Claim 47] method of claim 1, wherein the [neurological condition is] neuronal cell death occurs in a mammal having a neurological disease whereby glutamate or kainic acid mediated excitotoxicity is involved in neuronal cell death.

13. (Twice Amended) The [compound of Claim 47] method of claim 1, wherein the [neurological condition is] neuronal cell death occurs in a mammal having a neurological disease comprising Huntington's disease, Parkinson's disease or Alzheimer's disease.

14. (Four Times Amended) A method for assessing the ability of a compound to prevent neuronal cell death occurring in a mammal susceptible to or having a neurological condition, comprising:

a) contacting a compound with cultured neuronal cells having activated MLK activity, wherein activated MLK activity is selected from the group consisting of [an enzymatic activity] MLK1 activity, MLK2 activity, MLK3 activity, DLK activity, LZK activity, and an ability to bind a SEK1 protein [and an ability to phosphorylate a SEK1 protein];

b) contacting, in the presence of the compound, surviving cells from step (a) with an agent that induces apoptosis; and

(c) comparing the level of apoptosis in the cells in the presence of the compound with the level of apoptosis in the cells in the absence of the compound;  
wherein the compound is a potentially useful drug for treating mammals when the level of apoptosis in the cells in the presence of the compound is less than the level of apoptosis in the cells in the absence of the compound.

19. (Three Times Amended) A method for assessing a compound's ability to inhibit MLK activity, comprising:

- a) contacting a compound with a MLK protein and a substrate therefore, wherein the MLK protein is selected from the group consisting of MLK1, MLK2, MLK3, DLK, LZK and combinations thereof;
- b) measuring the level of MLK activity, wherein the MLK activity is selected from the group consisting of [an enzymatic] kinase activity and an ability to bind a SEK1 protein [and an ability to phosphorylate a SEK1 protein]; and
- c) comparing the level of MLK activity in the presence of the compound with the level of MLK activity in the absence of the compound, wherein a decrease in MLK activity in the presence of the compound is indicative that the compound has an ability to inhibit MLK activity.



## Molecular Cloning and Functional Expression of a cDNA Encoding a New Member of Mixed Lineage Protein Kinase from Human Brain\*

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Hiroyuki Sakuma†, Atsushi Ikeda†, Shogo Oka†, Yasunori Kozutsumi†, Jean-Pierre Zanetta§, and Toshisuke Kawasaki†¶

From the †Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan and §Laboratoire de Chimie Biologique Batiment C-9, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France

We have cloned a novel protein kinase from human cerebellum and named it LZK (leucine zipper-bearing kinase). The LZK cDNA encoded a 966-amino acid polypeptide that contains a kinase catalytic domain and double leucine/isoleucine zippers separated by a short spacer region. The amino acid sequence of the kinase catalytic domain was a hybrid between those in serine/threonine and tyrosine protein kinases, indicating that LZK belongs to the subfamily of the mixed lineage kinase (MLK) family. The kinase catalytic domain of LZK was most similar to DLK (Holtzman, L. B., Merritt, S.E., and Fan, G. (1994) *J. Biol. Chem.* 269, 30808-30817), MUK (Hirai, S., Izawa, M., Osada, S., Spyrou, G., and Ohno, S. (1996) *Oncogene* 12, 641-650), and ZPK (Reddy, U. R., and Presure, D. (1994) *Biochem. Biophys. Res. Commun.* 202, 613-620), which belong to the same subfamily of the MLK family. However, besides the kinase catalytic domain and double leucine/isoleucine zippers, there was no significant homology with known proteins. The recombinant LZK autophosphorylated in the presence of ATP and divalent cations, and exhibited serine/threonine kinase catalytic activity. Northern blot analysis revealed that LZK is expressed most strongly in the pancreas, with a pattern that differs from other MLKs. Expression of LZK in COS7 cells induced phosphorylation of c-Jun and activation of JNK-1, indicating the association of LZK in the c-Jun amino-terminal kinase/stress-activated protein kinase pathway. The expressed LZK was detected primarily in the membrane fraction, suggesting that LZK interacts with other cellular components *in vivo*.

Protein kinases play critical roles in the regulation of many cellular processes (1), such as the transmission of signals from growth factor (2, 3), control of cell growth and division (4), regulation of cytoskeletal changes (5), gene expression and differentiation (6), translation (7), and metabolism (1). The protein kinases can be divided into two groups based on their sequence similarities and their specificity for the acceptor

amino acid (1, 8, 9). Most protein kinases phosphorylate either serine/threonine or tyrosine, although protein kinases that modify histidine have been found. However, a small number of dual-specificity kinases can phosphorylate both serine/threonine and tyrosine residues (10), although they are structurally related to the serine/threonine-specific group. Protein kinases can also be grouped as receptor protein kinases and non-receptor protein kinases. Receptor protein kinases have an intracellular catalytic domain, transmembrane region, and extracellular ligand-binding domain. Protein kinases share, besides the protein kinase catalytic domain, some structural features reflecting their particular roles in protein-protein interactions. For example, the SH3<sup>1</sup> domains are found not only in tyrosine kinases and serine/threonine kinases but also in receptor-type and non-receptor protein kinases (11, 12). The leucine/isoleucine zipper sequence is found in some protein kinases (13). Recently, many new closely related intracellular kinases have been identified. One of these groups, mixed lineage kinases (MLKs), contains a unique double leucine/isoleucine zipper (14). MLK has a characteristic kinase catalytic domain with a sequence hybrid between those in serine/threonine and tyrosine protein kinases. These kinases include MLK1 (15), MLK2 (16, 17), MLK3/SPRK/PTK1 (18-20), and DLK/ZPK/MUK (21-23). Of these, DLK/ZPK/MUK are considered a secondary subfamily of MLK because of their characteristic sequences. However, little is known about the overall biochemical features and functional roles of MLKs.

We examined the cloning, expression, and preliminary characteristics of the novel intracellular protein kinase. The LZK cDNA encoded a protein with an apparent molecular mass of 135-150 kDa on reducing SDS-PAGE. Sequence analysis revealed that LZK belongs to the MLK family, containing the kinase catalytic domain and leucine/isoleucine zippers. However, LZK had no other strong homologies with any known proteins. The recombinant LZK protein exhibited serine/threonine protein kinase activities *in vitro*. Expression of LZK in COS7 cells induced phosphorylation of c-Jun and activation of JNK-1, suggesting the association of LZK in the JNK/SAPK pathway.

### EXPERIMENTAL PROCEDURES

*cDNA Library Screening and Sequence Determination of LZK*—A 826-bp rat cDNA clone with unknown functions, which had been iso-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB001872.

¶ To whom correspondence should be addressed. Tel.: 81-75-753-4572; Fax: 81-75-753-4605.

<sup>1</sup> The abbreviations used are: SH, Src homology domain; MLK, mixed-lineage kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; JNK, c-Jun amino-terminal kinase; SAPK, stress-activated protein kinase; JNKK, JNK kinase; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PVDF, polyvinylidene difluoride; bp, base pair(s); kb, kilobase pair(s); MEKK, MAPK/ERK kinase kinase; LZK, leucine zipper-bearing kinase; MUK, MAPK-upstream kinase; DLK, dual leucine zipper-bearing kinase; ZPK, leucine zipper protein kinase.

lated by screening of the  $\lambda$ ZAP rat brain cDNA library with an antibody raised against a soluble fraction of rat brain, was labeled with [ $\alpha$ - $^{32}$ P]dCTP by a random primer DNA labeling kit (TaKaRa), and the radiolabeled cDNA was used as a probe to screen approximately  $5 \times 10^6$  plaques of a human cerebellum cDNA library (CLONTECH). Hybridization was carried out in the buffer consisting of 50% formamide,  $5 \times$  SSC, 50 mM phosphate buffer, pH 7.0, 0.5% skim milk, 0.1% SDS, and 100  $\mu$ g/ml yeast RNA at 42 °C. Filters were washed at 65 °C in  $2 \times$  SSC containing 0.1% SDS and in  $0.2 \times$  SSC containing 0.1% SDS sequentially. Three unique clones were isolated and restriction mapped. The longest clone was subcloned into the plasmid vector Bluescript SK, sequenced along both strands over the entire length using a Taq DyeDeoxy terminator cycle sequence kit and an ABI 373A DNA Sequencer (Applied Biosystems).

**Analysis of LZK Transcript Expression**—Multiple human tissue Northern blot (CLONTECH) was hybridized to radiolabeled human LZK cDNA fragment (corresponding to nucleotides 1895–3174), which had been amplified by polymerase chain reaction and then labeled with [ $\alpha$ - $^{32}$ P]dCTP by a random primer method. Hybridization was performed as described above for cDNA screening. The filter was finally washed at 65 °C in  $0.1 \times$  SSC and 0.1% SDS, and analyzed by BAS 2000 image analyzer. To ensure the integrity and the quantity of RNA per lane, the blot was rehybridized to radiolabeled  $\beta$ -actin cDNA.

**Construction of Epitope-tagged LZK**—The cDNA fragment encoding the LZK open reading frame was engineered with XbaI restriction sites, and the product was amplified by long and accurate (LA)-polymerase chain reaction (oligonucleotides: 5'-GCTCTAGAATGGCCAACTTTCAGGAGCACCTGAGCTGCTCT-3'; 5'-GCTCTAGATCAATTACCAAGTAGCAGAGCTGTAGTGTATTGTT-3'). The digested fragment and a double-strand oligonucleotide linker (oligonucleotides: 5'-AGCTTCCACCATGAGAGGATCGCACCACCATCATCACCCT-3'; 5'-CTAGAGTGGTGTATGATGGTGGCAGTCTCTCATGGTGGGA-3') were inserted into the cytomegalovirus promoter-based eukaryotic expression vector pCDM8, which had been double-digested with HindIII and XbaI. The linker presented above was engineered with HindIII (5') and XbaI (3') restriction sites, and contained the typical Kozak's consensus sequence and coding sequence for the "MRGSHis<sub>6</sub>" epitope (Met-Arg-Gly-Ser-His<sub>6</sub>). The MRGSHis<sub>6</sub> tag was inserted into the amino terminus of the LZK coding sequence. The construct was sequenced to confirm Taq polymerase fidelity and maintenance of the appropriate reading frame.

**Expression of the LZK Constructs**—COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and kanamycin. Cells ( $2 \times 10^6$ ) plated onto a 10-cm tissue culture dish were grown overnight and transiently transfected with 10  $\mu$ g of the eukaryotic expression plasmid using LipofectAMINE™ (Life Technologies, Inc.) according to the manufacturer's protocol. After 48 h, cells were washed twice in ice-cold phosphate-buffered saline and then lysed by adding 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10% glycerol, 0.2% Triton X-100, and protease inhibitors). The lysate was sonicated on ice and then centrifuged for 20 min at  $105,000 \times g$  at 4 °C.

For immunoprecipitation, 2  $\mu$ g of anti-MRGSHis<sub>6</sub> antibody (QIAGEN) and 40  $\mu$ l of anti-mouse IgG-Sepharose (Sigma) (50% v/v) were added to the supernatant of the cell lysate, and the mixture was incubated at 4 °C overnight. Beads were washed five times in HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). For cell fractionation study, the cells were homogenized in the lysis buffer without Triton X-100, and the homogenate was centrifuged as above.

For immunoblot analysis, 20  $\mu$ l of cell lysate or the immunoprecipitates were separated under reducing conditions on a 7% SDS-polyacrylamide gel according to Laemmli (24). Proteins were electrically transferred onto nitrocellulose membranes, blocked for 2 h in Tris-buffered saline (TBS, pH 7.5) containing 3% nonfat dry milk, followed by incubation with the MRGSHis<sub>6</sub> antibody or the rabbit anti-LZK immune serum diluted 1:2000 in TBS containing 3% nonfat dry milk and 0.05% Tween 20, and then probed with appropriate horseradish peroxidase-conjugated second antibodies. Blots were developed using the chemiluminescent reagent (Pierce) and subjected to autoradiography.

**Detection of Kinase Activity in Vitro**—Immunoprecipitated MRGSHis<sub>6</sub>-LZK protein was washed four times with kinase assay buffer (25 mM Hepes, pH 7.2, 10% glycerol, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 0.1 mM sodium orthovanadate, and protease inhibitors). Immunoprecipitates were incubated in 50  $\mu$ l of kinase buffer containing 30  $\mu$ M ATP and 50  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol, Amersham) for 30 min at 30 °C, then resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membrane and then analyzed using

a BAS 2000 image analyzer.

**Phosphoamino Acid Analysis**—Phosphoamino acid analysis was carried out as described by Zheng and Guan (25). Following the *in vitro* kinase assay, the radioactive band of 135–150 kDa was excised from the PVDF membrane. The strip was incubated in 1 ml of 6 M HCl at 105 °C for 2 h. After removing of the strip, the sample was dried in a SpeedVac, then washed and dried twice in 1 ml of H<sub>2</sub>O. The resulting amino acids were separated on a cellulose plate by one-dimensional electrophoresis. Phosphoamino acid standards were visualized by ninhydrin staining, and radioactivity was detected by a BAS 2000 image analyzer.

**Immunoblot Analysis of c-Jun**—Cells ( $2 \times 10^6$ ) were transiently transfected with the expression vector harboring epitope-tagged LZK. After 48 h, cells were washed with ice-cold phosphate-buffered saline three times and then lysed *in situ* with 1 ml of Laemmli sample buffer. For control experiment, cells were stimulated by UV radiation (100 J/m<sup>2</sup>). After 1 h, they were used for the experiment. Cell lysate (20  $\mu$ l) was subjected to SDS-PAGE, and separated proteins were transferred on to the nitrocellulose membrane. The membrane was blocked by soaking in TBS containing 3% nonfat dry milk, incubated with diluted anti-c-Jun antibodies (0.1  $\mu$ g/ml in TBS containing 3% nonfat dry milk and 0.05% Tween 20), and subsequently with appropriately diluted horseradish peroxidase-conjugated secondary antibodies. The resulting membrane was developed using the chemiluminescent reagent and subjected to autoradiography.

**Detection of the JNK1 Activities**—Cells ( $2 \times 10^6$ ) were transiently transfected with the expression vector harboring epitope-tagged LZK. After 48 h, cells were washed three times with ice-cold phosphate-buffered saline and lysed with 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM NaF, 20 mM  $\beta$ -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin, and 2  $\mu$ g/ml pepstatin). After removal of insoluble materials by ultracentrifugation, 2  $\mu$ g of anti-JNK1 antibody (Santa Cruz Biotechnology) and 200  $\mu$ l of protein G-Sepharose (Sigma) (10% v/v) were added to the supernatant of the cell lysate, and the mixture was incubated at 4 °C overnight. Beads were washed four times in buffer consisting of 50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1% Triton X-100, 1 mM sodium orthovanadate, 50 mM NaF, 20 mM  $\beta$ -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin and 2  $\mu$ g/ml pepstatin, and subsequently washed four times in buffer consisting of 20 mM Hepes, pH 7.5, 15 mM MgCl<sub>2</sub>, 15 mM  $\beta$ -glycerophosphate, 0.1 mM sodium orthovanadate, and 2 mM DTT. The immunoprecipitates were incubated for 30 min at 30 °C in 30  $\mu$ l of the same buffer containing 25  $\mu$ M ATP, 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol, Amersham), and 2.5  $\mu$ g of glutathione S-transferase-c-Jun. Reactions were terminated by addition of the Laemmli sample buffer. The samples were boiled, resolved by SDS-PAGE, and then analyzed by using a BAS 2000 image analyzer.

## RESULTS

**Isolation of a LZK cDNA and Its Deduced Amino Acid Sequence**—A 826-bp rat cDNA fragment with unknown function was used as a probe to screen a human cerebellum cDNA library. Three independent clones were isolated, and their inserts were sequenced. The nucleotide sequence of the longest insert is shown in Fig. 1. The cDNA extends over 3450 nucleotide bases and contains 272 bp of 5'-untranslated nucleotides, a continuous open reading frame of 2898 bp, and 399 bp of 3'-untranslated nucleotides. The putative initiation codon was assigned at nucleotide 273. This methionine codon is located within a sequence context favorable for the Kozak's rule and is preceded by an in-frame stop codon beginning at base 234. Within the 3'-untranslated region, putative polyadenylation signals are found at 3318 bp (AATAA), at 3354 bp (AATAA), at 3370 bp (AATTAA), and at 3517 bp (AATAA) upstream from the poly(A) tract. The longest open reading frame of the cDNA encodes a putative polypeptide of 966 amino acids, with a calculated molecular mass of 108 kDa. Hydrophobicity analysis revealed that the protein contains no obvious signal sequence or transmembrane domain (data not shown). Comparison of the sequence with other known proteins revealed that the protein can be divided into several structural domains: a kinase catalytic domain, a double leucine/isoleucine zipper separated by a short spacer region, and an acidic domain at its carboxyl-

FIG. 1. The nucleotide and deduced amino acid sequences of human LZK cDNA. Nucleotide and amino acid numbers are indicated at the left-hand side of each lane. An asterisk (\*) denotes the position of the in-frame stop codon located upstream of putative initiation methionine. Putative locations for the polyadenylation signal sequence are underlined. Arrows denote boundaries of the kinase catalytic domain. Hydrophobic residues occupying the *d* positions in putative leucine/isoleucine zipper domains are circled. The completely conserved amino acid sequence (SSEEEEGEVDSEVE) commonly found in carboxyl-terminal domain of LZK and ZPK/DLK/MUK is boxed.

Following the kinase catalytic domain, LZK contained two

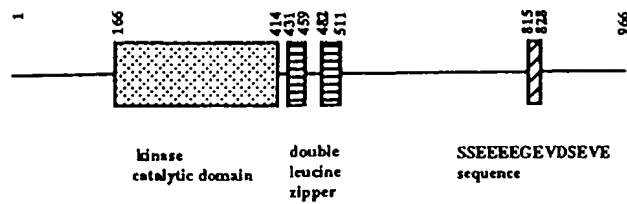


FIG. 2. Schematic representation of human LZK primary structure. The numbers above the diagram represent amino acid residue number and delineate boundaries of indicated domains (motifs). The kinase domain (stippled box), leucine/isoleucine zipper domains (striped box), and the SSEEVEGEVDSEVE sequence (slash) are shown. Between two leucine/isoleucine zippers, there is a spacer region.

heptad repeats of nonaromatic hydrophobic amino acids separated by a 25-amino acid spacer. By Chou and Fasman analysis (32), this amino acid sequence formed an  $\alpha$ -helix structure, indicating that these regions of LZK are composed of two leucine/isoleucine zipper motifs (Figs. 2 and 3), which may promote homo- or heterodimerization of proteins through hydrophobic interactions. As shown in Fig. 4, hydrophobic residues are conserved at the *d* position in zipper 1 and 2, forming a hydrophobic stripe on the face of the helix. Except for the *d* position, these regions are comparatively rich in charged amino acids. In particular, position *b* (EETE) and position *f* (KSRR) in zipper 1, and position *g* (IRRK) in position 2 were primarily composed of negatively or positively charged amino acids, suggesting that they are involved in intra- or intermolecular electrostatic interactions (33, 34).

The regions containing the kinase catalytic domain and leucine zipper domain of this protein have 86.4% and 86.4% identity, respectively, to previously reported proteins DLK (dual leucine zipper-bearing kinase) (21) and ZPK (leucine zipper protein kinase) (22) (see Fig. 3). In addition, the sequence of this region was homologous to MLK1 (15), MLK2 (16, 17), and MLK3 (18–20) by 40.2%, 40.4%, and 39.5%, respectively (Figs. 3 and 5), suggesting that LZK, together with DLK/ZPK, belongs to the MLK (mixed lineage kinase) family, although no strong similarity was found outside this region. However, in contrast to the other of MLKs, which have a SH3 domain at their amino-terminal ends, LZK (as well as DLK/ZPK) did not contain such a structure (Fig. 5). In addition, LZK and DLK/ZPK have a single invariant Glu at 7 amino acid residues downstream from the invariant Lys in subdomain II, but this is not the case with ordinary MLKs. This Glu residue is believed to play an important role in stabilizing ATP in the ATP-binding site from the crystallographic study (35). These results suggest that LZK, together with DLK and ZPK, belongs to the secondary subgroup of MLK. In addition, LZK and DLK/ZPK share a unique sequence, Ser-Ser-Glu-Glu-Glu-Gly-Glu-Val-Asp-Ser-Glu-Val-Glu (Ser<sup>615</sup>-Glu<sup>628</sup> in LZK) (Figs. 5 and 6). However, the glycine/proline-rich region present in DLK/ZPK at the carboxyl- and amino-terminal ends was not detected in LZK (Fig. 5). It should be noted that the sequence of the LZK kinase catalytic domain is 94.6% identical with that of a partial putative serine/threonine protein kinase (36), implying that these proteins are identical or closely related (Fig. 3).

**Tissue Distribution of LZK mRNA**—Expression of LZK mRNA was examined by Northern blotting mRNA from several human tissues. The probe used for this analysis was corresponded to nucleotides 1895–3174 (See Fig. 1). Three bands at about >9.5, 8.7, and 6.5 kb were found with pancreas mRNA at the highest level. These bands were also markedly detected in the brain, liver, and placenta, and no positive signal was detected in the heart, lung, skeletal muscle or kidney (Fig. 7A). The expression levels of these three transcripts varied among the tissues. The 8.7-kb band was detected only in mRNA from

pancreas. Similarly, the >9.5-kb band was detected only with pancreas and brain. After initial probing with LZK cDNA, the blot was rehybridized with  $\beta$ -actin cDNA to confirm the integrity of the RNA from different tissues (Fig. 7B).

**Expression of LZK cDNA in COS 7 Cells and in Vitro Phosphorylation of the Recombinant LZK**—To facilitate the detection and immunoprecipitation of the LZK, MRGSHis<sub>6</sub> epitope was incorporated at the amino terminus of LZK (see "Experimental Procedures"). The epitope-tagged full-length LZK cDNA was incorporated into the eukaryotic expression vector pCDM8, and the resulting plasmid was transfected into COS 7 cells. Upon immunoblot analysis of LZK transfectants following the SDS-PAGE under reducing conditions, a protein with a molecular mass of 135–150 kDa, which is in good agreement with the predicted mass of the epitope-tagged LZK, was detected, while no band was detected for the non-transfectant (Fig. 8A). In addition, a protein of 135–150 kDa was specifically immunoprecipitated with a MRGSHis<sub>6</sub> antibody from the lysate of the transfectant (data not shown).

To study the subcellular localization of LZK, COS7 cells expressing LZK were homogenized in the absence of detergent. The homogenate was fractionated into the soluble and the membrane fractions, and the respective fractions were subjected to SDS-PAGE and followed by immunoblot analysis. Strong immunoreactive bands were detected in the membrane fraction, while only weak bands were found in the soluble fraction (Fig. 8B), suggesting that LZK protein binds to some membrane components probably through interaction with some other cellular components such as lipid and/or anchor protein.

To confirm that LZK is an active protein kinase, MRGSHis<sub>6</sub> antibody immunoprecipitates of the LZK transfectants were incubated with [ $\gamma$ -<sup>32</sup>P]ATP in the presence of Mn<sup>2+</sup>, Mg<sup>2+</sup>, and Na<sub>3</sub>VO<sub>4</sub> (protein-tyrosine phosphatase inhibitor), and then the proteins were separated by SDS-PAGE under reducing conditions followed by transfer onto PVDF membranes. Upon autoradiography, immunoprecipitates from the transfectants revealed radioactive bands of 135–150 and 50 kDa, but no detectable bands in non-transfectants (Fig. 9B). The radioactive band of 50 kDa comigrated with the band of heavy chain of IgG, indicating that LZK not only autophosphorylated itself but also phosphorylated heavy chain of IgG.

The radioactive 135–150-kDa band of LZK from the *in vitro* kinase assay was excised and subjected to partial acid hydrolysis. The resulting materials were separated by one-dimensional electrophoresis on a cellulose plate (25). Analysis by autoradiography and comparison to ninhydrin-stained phosphoamino acid standards revealed only phosphoserine and phosphothreonine (Fig. 9C), indicating that LZK has a serine/threonine kinase activity. However, the present experiment cannot completely exclude the possibility that LZK has a tyrosine kinase activity.

**Activation of JNK Pathway by LZK**—Recent studies show that some MLKs activate JNK pathway (23, 26, 27). JNK pathway is believed to be predominantly activated by cellular stresses such as UV radiation, inflammatory cytokines, and osmotic shock (28, 29), which results in the activation of transcriptional factors such as c-Jun and ATF2 (30, 31). Because the amino acid sequence of LZK showed high homology to DLK/MUK, which were known to activate JNK pathway, we tested whether or not LZK activates the phosphorylation of c-Jun. COS7 cells were transiently transfected with the expression vector harboring an epitope-tagged LZK, after which the mobility delay of endogenous c-Jun was monitored by immunoblot analysis with anti-c-Jun antibodies. As shown in Fig. 10, expression of LZK induced the mobility delay of c-Jun as much

		I	II	III	IV
LZK	165	FEKSELQMLGSAQGAATLCKFRATFVAIKKVRQNETD-----	IKGLAKLKHFNII		
ZPK	122	...LD...V.....R.HG...V...DLK.....			
DLK	155	...LD...V.....R.HG...V...DLK.....			
MUK	155	...LD...MV.....HG...V...KDLK.....			
MLK1	1	A.LTLKEII.I...F.K.YRAFWIGD...V.AA.HDPDE.ISQTIENVRQEA.LFAM.....			
MLK2	95	H.LQLKEII.V...F.K.YRALWIG...V.AA.LDP.F.PAVTAEQVCQEARLPGA.Q.....			
MLK3	114	Q.LALEEVI.I...F.K.YR.SWRG.L...V.AA.QDPDE.ISVTAEVSRQEARLPGA.A.....			
		V	Via		
LZK	218	AFKGVCTQAPCYCIIMEYCAHQQLYEVLRAGRKITPRLVDWSTGSIASGHNYLH---	LMKIIHR		
HsPK37	1	FLY.....E.....			
ZPK	175	T.....F..Q.....PV.....M...G.....			
DLK	208	T.....L..F..Q.....PV..S.....M...G.....			
MUK	208	T.....L..F..Q.....PV..S.....M...G.....			
MLK1	64	.LR...LKE.NL.LV...FARG.F.NR...S.KR.F.DI..N.AVQ..R.....DEAIVP...			
MLK2	159	.LR.A.LNP.NL.LV...ARG.A.SR...RVP.HV..N.AVQV.R.....NDAPVP...			
MLK3	178	.LKA...LKE.NL.LV...AAG.F.SRA...RVP.HV..N.AVQ..R...M...CEA.VPV...			
		Vib	VII	VIII	
LZK	279	DLKSPHVLV-----THTDAV-KISDFGTSKELSDKSTKMSFAGTVAMHAFEVIRNEPVSXV			
HsPK37	52	.....			
ZPK	236	.....M.I-----YD.V.-.....			
DLK	269	.....M.I-----YD.V.-.....			
MUK	269	.....M.I-----YD.V.-.....			
MLK1	127	...S.I..ILQKVENGLSNXIL..T...LAR.-WHRT...A.....ASMF.KGS			
MLK2	222	...I.I..ILKAIENHGLA.T.L..T...LAR.-WH.T...A.....LSLF.KSS			
MLK3	241	...N.I..LQPIESDMDKDTL..T...LAR.-WH.T.Q..A.....KASTF.KGS			
		IX	X	XI	
LZK	335	DIWSTGVVLWELLTGEIPIYKDVDSALIMGVGSHSLHLPVPSSTCPDGEKILHQAQWQSKFANRF			
HsPK37	106	....MV.....			
DLK	292	.....S.....LR.C.N.....			
ZPK	325	.....S.....LR.C.N.....			
MUK	325	.....S.....LR.C.NR.....			
MLK1	190	.V..Y..L.....V.FRG.I.GLRVAY..AM.K.A..I....EP.AK..EDC.NPD.HS..			
MLK2	285	.V.....L.....V..REI.AL.VAY..AM.K.T..I....EP.AR.LEEC.DPD.HG..			
MLK3	304	.V.....L.....V..RGI.CL.VAY..AV.K.T..I....EP.AQ..ADC.AQD.HR..			
		zipper 1			
LZK	399	SFRQTLQGLD-IASADVLTAPQETIFKSAQEWREEVKKGFKEIKSEGTCTIKRLDEKLIRAREE			
DLK	356	...I.L...-.....S.....L.....L...E...M....			
ZPK	389	...I.L...-.....S.....L.....L...E...M....			
MUK	389	...I.L...-.....S.....L.....L...E...VM....			
MLK1	254	..TNI.DQ.TT.EKSGFFEM.KDSFMCL.DN.KH.IQEM.DQLRAKEXELRTWE...T.AALQQ			
MLK2	349	D.GSI.KR.FV.EQSALPOM.L.SFMEL.ED.KL.IQHM.DDLRTKCKELRSRE...L.AAQ.Q			
MLK3	368	D.ASI.QQ.EALEAQVLRHM.RDSPMNM.EG.KR.IQGL.DELRAKCKELLSRE...T.AA..Q			
		zipper 2			
LZK	462	LRHALDIREHYERKLRANMLYKLSAHLQLDREKELIKREQAVEKY			
ZPK	419	.....N.L.....LK.R..LR...L.RRC			
DLK	452	.....N.L.....LK.R..LR...L.RRC			
MUK	452	.....N.L.....LK.R..LR...L.RRC			
MLK1	318	KMQ-----EEL.RRREQE.AE..IDILE..LNIIHQ			
MLK2	413	RFG-----EQQ.RRREQE.AE..MDIVE..LMELMCQ			
MLK3	432	RSQ-----AEQ.RRREHL.AQM.L.VFE..LTLLLQQ			

FIG. 3. Sequence alignment of kinase catalytic domains and leucine/isoleucine zipper domains of the MLK protein kinase family. Alignment of the LZK catalytic domain and leucine zipper domains with those of putative protein kinase (partial), ZPK, DLK, MUK, MLK1 (partial), MLK2, and MLK3. Amino acids are numbered at the left. Amino acids identical with LZK are shown by dots. Conserved kinase subdomains are numbered with Roman numerals. Putative leucine/isoleucine zippers are indicated above the line, and the hydrophobic amino acids located at the d position of each putative zipper are denoted with a d.

as was observed with UV radiation. Because the mobility delay is caused by the phosphorylation of c-Jun, these results suggested that expressed LZK activates the endogenous JNK pathway (28). Then to confirm that the phosphorylation of c-Jun observed was really caused by activation of JNK, endogenous JNK1 was immunoprecipitated from the cell lysate and JNK1 activity was determined by *in vitro* kinase assay using soluble glutathione S-transferase-c-Jun as substrate. As shown in Fig. 11, expression of LZK elevated the JNK1 kinase activity. The

extent of JNK1 activation by expression of LZK was comparable to that caused by UV radiation. These results taken together indicated that LZK can effectively activate JNK pathway.

#### DISCUSSION

We examined the cDNA cloning, expression, and characteristics of a novel protein kinase, which is expressed in a spatially regulated fashion in adult human tissues. This protein kinase

FIG. 4. Helical wheel representation of the leucine/isoleucine zippers of LZK. The residues of putative leucine/isoleucine zippers of LZK were arrayed on a helical wheel. The spokes of the wheel show the relative positions of the amino acids in an  $\alpha$ -helix, and the positions *a*–*d* correspond to the location of the amino acid residues. In an ideal  $\alpha$ -helix, amino acid residues appear on one side of the helix in every two turns. In this model, conserved hydrophobic amino acids were located at the *d* position.

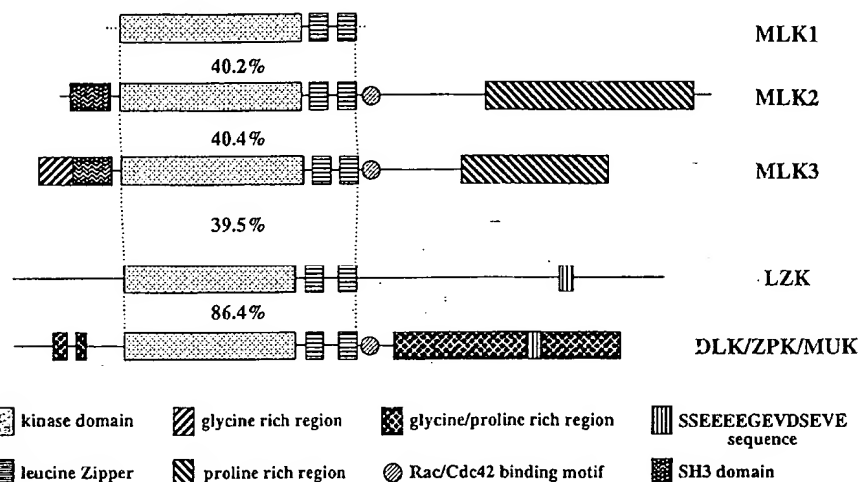
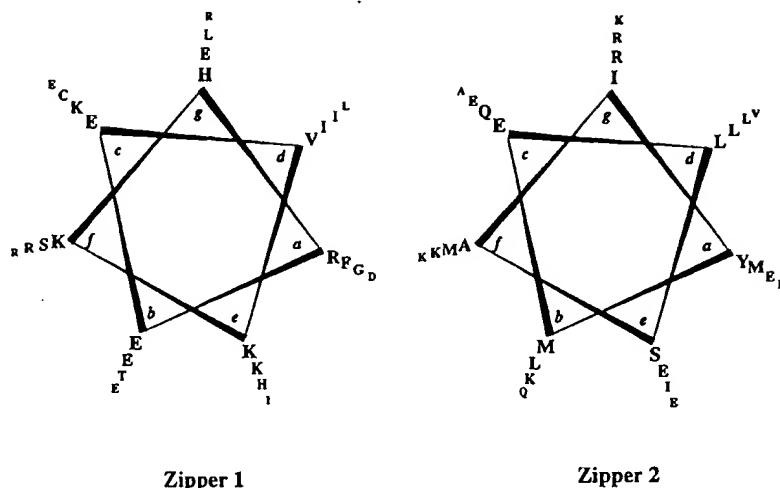


FIG. 5. Schematic representation of the structures of LZK and other MLKs. SH3 domains, glycine-rich regions, proline-rich regions, glycine/proline-rich regions, kinase domains, leucine zippers, Rac/Cdc42 binding motifs, and SSEEVEGEVDSEVE sequences are shown.

H-LZK	805	TRPLQKSGDDSSSEEVEGEVDSEVEFPRQRPHRCISS
ZPK (human)	718	AVTRSQRKRGISSEEVEGEVDSEVELTSSQRWQPSLNM
DLK (mouse)	735	AVTRSQRKRGISSEEVEGEVDSEVELPPSQRWPGPNM
MUK (rat)	741	AVTRSQRKRGISSEEVEGEVDSEVELPPSQRWPGPNM

FIG. 6. The amino acid sequence SSEEVEGEVDSEVE were commonly conserved in the carboxyl-terminal domain of LZK and ZPK/DLK/MUK. Besides the regions of kinase catalytic domain and leucine/isoleucine zippers, LZK does not show strong similarity with other protein kinases of the MLK family (see the text). However, the short amino acid sequence SSEEVEGEVDSEVE is completely conserved in the carboxyl-terminal domain of LZK and DLK/ZPK. The amino acid numbers are indicated on the left side of the sequences.

contains a kinase catalytic domain, followed by two leucine/isoleucine zipper motifs, which are separated by a short spacer region. We designated this protein kinase as LZK. The LZK cDNA encodes a protein with an apparent molecular mass of 135–150 kDa, and has serine/threonine kinase activity.

LZK is most similar to DLK and ZPK. DLK was identified by Holzman (21) as a novel protein kinase with a unique kinase catalytic domain, the expression of which is regulated spatially and developmentally. ZPK is cloned and identified as a novel putative protein kinase, which is up-regulated in retinoic acid-treated NT2 cells (22). When the region containing the kinase catalytic domain and the leucine/isoleucine zipper domain of LZK was aligned to DLK and ZPK, homology was 86.4% and 86.4%, respectively, with no insertion and/or deletion. Like

DLK and ZPK, LZK had invariant Glu at the specific location 7 amino acids downstream from invariant Lys in subdomain II. From crystallographic study and structure-function analysis of other protein kinases, the invariant Glu in subdomain III and invariant Lys in subdomain II are believed to play an important role in stabilizing ATP in the ATP-binding site.

The amino acid sequence WMAPE in subdomain VIII is often found in Raf family protein kinases, suggesting that LZK has a MAPKKK-related activity. It is interesting to note that Hirai *et al.* (23) recently identified MUK, which corresponds to rat homologue of DLK (mouse) and/or ZPK (human), as a MAPKKK-related protein kinase such as c-Raf and MAPK/ERK kinase kinase (MEKK) (37). They showed that MUK phosphorylates and activates JNKK *in vivo* and *in vitro*. JNKK (38, 39) can be phosphorylated and activated by the MAPKKK-related kinase, MEKK (40, 41), and acts on Jun kinases, resulting in activation of c-Jun (29, 42). MUK-transfected cells induced hyperphosphorylation of c-Jun, suggesting that MUK can regulate the JNK/SAPK pathway *in vivo*. The induction of JNK was also observed in a truncated MUK consisting of the kinase catalytic domain and leucine/isoleucine zipper motifs, the amino acid sequence of which was 86.4% identical to that of LZK. As might be expected from this high homology with MUK, LZK was in fact shown to induce phosphorylation of c-Jun and activation of JNK1, indicating that LZK stimulates the JNK/SAPK pathway. The extent of JNK1 activation by LZK expression was comparable to that caused by UV radiation. Consid-



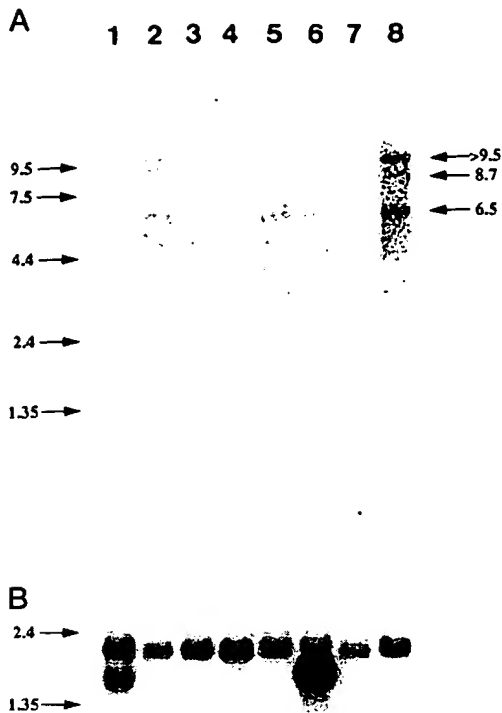


FIG. 7. Expression of LZK mRNA in adult human tissues. The Northern blot was purchased from CLONTECH. In each lane, 2  $\mu$ g of poly(A)<sup>+</sup> RNA from human tissues were loaded. A, the blot was hybridized to a radiolabeled probe corresponding to LZK nucleotides 1895–3174. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. B, the blot was rehybridized with a radiolabeled actin probe to confirm the integrity of the RNA.

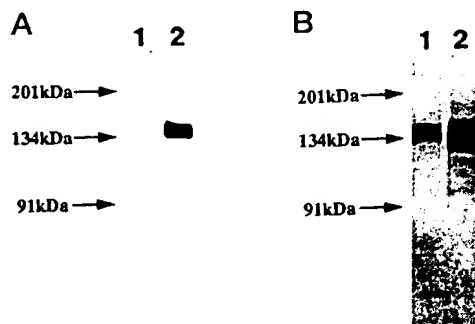


FIG. 8. Expression and cellular localization of epitope-tagged LZK in COS7 cells. A, the MRGSHis<sub>6</sub>-tagged LZK was transiently transfected into COS7 cells. Transfectants and non-transfectants were lysed in the presence of detergent (see "Experimental Procedures"), and the lysate was resolved by SDS-PAGE under reducing conditions, followed by immunoblot analysis with a MRGSHis<sub>6</sub> antibody. Lane 1, non-transfectant; lane 2, transfectant. B, COS7 cells transiently transfected with epitope-tagged LZK were lysed in detergent-free lysis buffer (see "Experimental Procedures") and fractionated by ultracentrifugation. The resulting supernatant (soluble fraction) and pellet (insoluble fraction) were resolved by SDS-PAGE under the reducing conditions, followed by immunoblot analysis with MRGSHis<sub>6</sub> antibody. The loaded materials in each lane corresponded to the equal numbers of the cells. Lane 1, soluble fraction; lane 2, insoluble fraction.

ering the efficiency and cytotoxicity of the transfection procedure, it seems reasonable to speculate that LZK directly phosphorylates and activates the main components of JNK pathway, such as JNKK and MEKK *in vivo*.

When expressed in COS7, LZK was present in both cytosol and membrane fractions. Because LZK contains no obvious



FIG. 9. Immunoprecipitation, autophosphorylation, and phosphoamino acid analysis of LZK. A, expressed epitope-tagged LZK was immunoprecipitated from cell lysate as described under "Experimental Procedures." Immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis with MRGSHis<sub>6</sub> antibody. Lane 1, non-transfectant; lane 2, transfectant. B, immunoprecipitates were incubated in kinase assay buffer containing [ $\gamma$ -<sup>32</sup>P]ATP and divalent cations, subjected to SDS-PAGE, transferred onto the PVDF membrane, and analyzed by BAS 2000. Lane 1, immunoprecipitates from non-transfectant; lane 2, immunoprecipitates from LZK-transfected cells. C, radioactive band of 130–140 kDa that corresponds to autophosphorylated LZK was excised and subjected to partial acid hydrolysis. Resultant material was resolved by one-dimensional electrophoresis. pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; Pi, free phosphate.

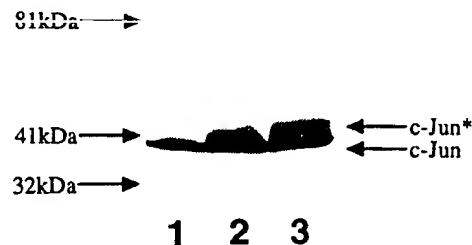


FIG. 10. Hyperphosphorylation of c-Jun by LZK. Cells were lysed with the Laemmli sample buffer and then subjected to SDS-PAGE under reducing conditions, followed by immunoblot analysis with anti-c-Jun antibodies. Lane 1, control cells (non-transfectants); lane 2, cells stimulated by UV radiation (100 J/m<sup>2</sup>); lane 3, cells transfected with pCDM8-LZK.

signal sequence or transmembrane domain, LZK should first be synthesized in cytosol and then translocated to membranes. It has been thought that subcellular compartmentalization is crucial in providing specificity in the regulation and function of protein kinases (43). Some protein kinases were targeted in a given compartment in the cell, and following various stimulations, they translocated to new sites within the cell, where they associated with anchor proteins, regulated by other protein and/or lipid, to gain access to their physiological substrates. Mata *et al.* (44) recently reported that DLK also exists in both cytosolic and membrane-bound form. They showed that each form of DLK has different biochemical characteristics. The membrane-bound form of DLK is not phosphorylated and forms high molecular complexes, and the cytosolic form of DLK is phosphorylated and exists as monomers. Since LZK, unlike other related protein kinases, does not contain a SH3 domain or a proline-rich region that is a presumed SH3-binding motif, it remains to be clarified what kind of interaction induces the translocation of LZK and regulates the functions of LZK. Recently, MLK-3 was shown to interact specifically with the GTP-bound form of Rac and Cdc42 (45), which regulates the JNK signaling pathway leading to the c-Jun post-transcriptional

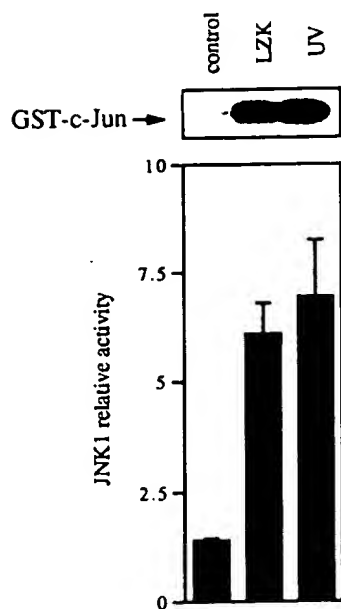


FIG. 11. Activation of JNK-1 by LZK. Endogenous JNK1 was immunoprecipitated from COS7 cells and incubated with glutathione S-transferase-c-Jun in the presence of [ $\gamma$ - $^{32}$ P]ATP (see "Experimental Procedures"). The reaction was stopped by the addition of the Laemmli sample buffer, and the samples were separated by SDS-PAGE and then analyzed by using the BAS 2000 image analyzer. The data shown in the upper panel were quantified and are shown in the graph. Data represent the mean  $\pm$  S.E. of three independent experiments and are expressed as JNK1 relative activity (lower panel).

activation (46–48). Considering that structurally related MUK activated the JNK pathway, LZK might be associated with mitogen-activated protein kinase pathways under the regulation of a small GTP-binding protein. However, to clarify the mechanism which might regulate the function of LZK, further studies must be done on the biochemical difference between phosphorylated and non-phosphorylated forms of LZK and the signals regulating the compartmentalization of LZK.

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# The Mixed Lineage Kinase DLK Utilizes MKK7 and Not MKK4 as Substrate\*

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Steven E. Merritt†, Marina Mata§, Deepak Nihalani†, Chongxue Zhu§, Xiaoping Hu§, and Lawrence B. Holzman†||

From the †Department of Veterans Affairs, Ann Arbor, Michigan 48105, the ‡Division of Nephrology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, 48109-0676, the §Department of Neurology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, and the Department of Veterans Affairs, Pittsburgh, Pennsylvania 15213

Mixed lineage kinases DLK (dual leucine zipper-bearing kinase) and MLK3 have been proposed to function as mitogen-activated protein kinase kinases in pathways leading to stress-activated protein kinase/c-Jun NH<sub>2</sub>-terminal kinase activation. Differences in primary protein structure place these MLK (mixed lineage kinase) enzymes in separate subfamilies and suggest that they perform distinct functional roles. Both DLK and MLK3 associated with, phosphorylated, and activated MKK7 *in vitro*. Unlike MLK3, however, DLK did not phosphorylate or activate recombinant MKK4 *in vitro*. In confirmatory experiments performed *in vivo*, DLK both associated with and activated MKK7. The relative localization of endogenous DLK, MLK3, MKK4, and MKK7 was determined in cells of the nervous system. Distinct from MLK3, which was identified in non-neuronal cells, DLK and MKK7 were detected predominantly in neurons in sections of adult rat cortex by immunocytochemistry. Subcellular fractionation experiments of cerebral cortex identified DLK and MKK7 in similar nuclear and extranuclear subcellular compartments. Concordant with biochemical experiments, however, MKK4 occupied compartments distinct from that of DLK and MKK7. That DLK and MKK7 occupied subcellular compartments distinct from MKK4 was confirmed by immunocytochemistry in primary neuronal culture. The dissimilar cellular specificity of DLK and MLK3 and the specific substrate utilization and subcellular compartmentation of DLK suggest that specific mixed lineage kinases participate in unique signal transduction events.

A large body of work has focused on signal transduction via protein kinases generically termed mitogen-activated protein kinases (MAPK)<sup>1</sup> that link a variety of extracellular signals to

cellular responses as diverse as proliferation, differentiation, and apoptosis (reviewed in Refs. 1–3). Biochemical and genetic evidence has demonstrated that activation of a prototypical MAPK occurs through sequential activation of a series of upstream kinases: a serine/threonine MAPK kinase kinase (MAPKKK) phosphorylates a dual specificity protein kinase (MAPKK or MKK or MEK) that in turn phosphorylates and activates a MAPK. Three groups of mammalian MAPKs and the upstream kinases and stimuli that activate them have been studied most extensively. These include the p42/p44<sup>MAPK</sup>s (extracellular signal-regulated kinases, ERK1 and ERK2), that are generally activated by mitogens and differentiation inducing stimuli, the p46/p54<sup>SAPK</sup>s (stress-activated protein kinases, SAPKs), and the p38<sup>MAPK</sup>s. Stress-activated protein kinases were discovered as the principal c-Jun NH<sub>2</sub>-terminal kinases and therefore have also been termed JNKs. Distinct from ERK1 and ERK2, the SAPKs are predominantly activated by cell stress-inducing signals such as heat shock, ultraviolet irradiation, proinflammatory cytokines, hyperosmolarity, ischemia/reperfusion, and axonal injury.

Like previously identified MAPK pathways in mammalian cells and yeast, the SAPK pathways were initially thought to lead in a linear fashion from activation of a Rho-like small GTPase through a series of intermediate protein kinases to SAPK activation. However, with the identification of two MAPKKs (MKK4/SEK1, MKK7/JNKK2) many MAPKKs (four MEKKs, five MLKs, ASK1, Tpl-2, and TAK1) and multiple additional MAPKKK kinases that appear to lie in pathways proximal to the SAPKs, it is clear that the organization, regulation, and function of these protein kinase pathways remain poorly understood.

The five identified members of the mixed lineage kinase (MLK) family share two common structural features (4, 5). Each has a kinase catalytic domain whose primary structure is hybrid between those found in serine/threonine and tyrosine protein but most closely resemble MAPK kinase kinases. Second, closely juxtaposed COOH-terminal to the catalytic domain, each MLK protein has a domain that is predicted to form two leucine/isoleucine zippers separated by a short spacer region. It has been proposed that all members of the MLK family behave functionally as MAPK kinase kinases in pathways leading to activation of SAPKs (6). In support of this hypothesis, MLK2, MLK3, DLK, and LZK have all been shown to activate p46<sup>SAPK</sup> when overexpressed in cultured cells (6–12). Furthermore, MLK2 and MLK3 have been shown to associate with, phosphorylate, and activate MKK4 (6, 12, 13).

Despite the common features of the MLK family, the various members of the family are likely to have diverse biological behavior. This is predicted by comparison of structure that shows that MLK1, MLK2, and MLK3 form one closely related

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|| To whom correspondence should be addressed: University of Michigan Medical School, 1560 Medical Science Research Bldg. II, Ann Arbor, MI 48109-0676. Tel.: 734-764-3157; Fax: 734-763-0982; E-mail: lholzman@umich.edu.

<sup>1</sup> The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK (also MEK and MKK), mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; SAPK, stress-activated protein kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MLK, mixed lineage kinase; DLK, dual leucine zipper-bearing kinase; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

MLK subfamily while DLK and LZK form a second distinct subfamily (5, 11). MLK2 and MLK3 have highly conserved kinase catalytic and leucine zipper domains, sharing more than 70% sequence identity. Both possess an SH3 domain in their NH<sub>2</sub>-terminal region, and both have a functional CRIB domain that mediates GTP-dependent association with Rac1 and Cdc42Hs (9, 14). DLK and LZK share highly conserved kinase catalytic and leucine zipper domains that are more than 90% identical, whereas these domains have only 36% identity to those of MLK2 and MLK3. Given the noted structural differences, DLK and LZK substrates may be distinct from those utilized by MLK2 and MLK3. Indeed, DLK and LZK substrates have not been identified. Moreover, DLK and LZK lack both CRIB motifs and SH3 domains and possess COOH-terminal regions that are structurally distinct from those of MLK2 and MLK3. These observations predict that protein-protein interactions unique to each protein kinase lead to distinct regulation and function by determining different subcellular compartmentalization and by providing access to different substrates.

The studies reported here provide evidence in support of this hypothesis. *In vitro* and complementary *in vivo* biochemical studies demonstrated that both DLK and MLK3 directly associate with, phosphorylate, and activate MKK7. Unlike MLK3, however, DLK did not phosphorylate or activate recombinant MKK4 *in vitro*. To investigate the physiological relevance of these observations, the cellular and subcellular localization of endogenous DLK, MLK3, MKK7, and MKK4 was investigated both in neuron cell culture and in the nervous system. Whereas MLK3 was identified predominantly in the periventricular ependyma and meninges, DLK and MKK7 were predominantly localized in neurons. DLK and MKK7 were identified in similar nuclear and extranuclear compartments. However, MKK4 occupied compartments distinct from that of DLK and MKK7. The dissimilar cellular and subcellular compartmentalization and differential substrate utilization of DLK and MLK3 provide evidence that specific mixed lineage kinases participate in unique signal transduction events.

#### MATERIALS AND METHODS

**Antibodies**—A rabbit polyclonal antiserum directed against the carboxyl-terminal 223 amino acids of DLK (C1) was described previously (5). Affinity purified rabbit anti-MKK7 polyclonal serum was a gift of Professor Eisuke Nishida (15). Monoclonal antibodies against the FLAG epitope (M2, Kodak/IBI), the Myc epitope (9E10, Oncogene Science), and the hemagglutinin epitope (12CA5, BMB) were obtained commercially as were rabbit polyclonal antibodies against SAPK/JNK3 (Upstate Biotechnology, Inc.), MLK3 (Santa Cruz), phospho-MKK4 (New England Biolabs), and MKK4 (Upstate Biotechnology, Inc.). Mouse monoclonal antibodies for SAPK/JNK1 (Pharmagen) were obtained commercially. In immunoblotting experiments, GST-MKK proteins were detected using the C1 polyclonal serum that was raised against a GST-DLK fusion protein.

**Plasmids and Plasmid Constructions**—Previously described plasmids used herein included DLK(K185A) and DLK (16), MEKK4 and MKK4-KM (Ref. 17, gifts of Dr. G. Johnson), FLAG-MLK3, FLAG-MLK3(K144E) (Ref. 12, gifts of Dr. J. Woodgett), GST-MKK1 and GST-MKK3 (Ref. 18, gifts of Dr. K.-L. Guan), GST-MKK5 (Ref. 19, gift of Dr. J. Dixon), GST-MKK7 (Ref. 20, gift of Dr. R. Davis), Myc-p46<sup>SAPK</sup> (7), and GST-c-Jun (1-79) (7). GST-MKK7(K76A) was created by introducing point mutations into the GST-MKK7 template using sequential polymerase chain reaction steps as described (16). Synthetic oligonucleotides used included 1) a 5' MKK7 oligonucleotide 5'-CCCGGATC-CATGCTGGGGCTCCCATCA-3', 2) a 3' MKK7 antisense oligonucleotide 5'-GCCGAATTCCTACCTGAAGAAGGGCAG-3', and 3) 5'-CATTGCTGTTGCGCAATGCG-3' and 4) 5'-CGCATTTGCGCAACAGCAATG-3' as antisense and sense K-A mutagenesis oligonucleotides, respectively. The BamHI-EcoRI fragment of the resultant amplification product was subcloned into the BamHI-EcoRI prepared PGEX-KT plasmid (21). FLAG-MKK7 and FLAG-MKK7-KA expression constructs were prepared using polymerase chain reaction employing Expand High-Fidelity DNA polymerase (Boehringer Mannheim), GST-MKK7 or GST-MKK7(KA) plasmid as templates, and synthetic oligonucleotides, including a 5' oligonucleotide encoding a Kozak's consensus and a FLAG-epitope (5'-ATAAAGCTTCCAGAGGCCATGGACTACAAGGACGACG-ATGACAAGCTGGGGCTCCCATCAACCTTGTTC-3'). Amplified fragments were subcloned into pCR3.1 (Invitrogen) following the manufacturer's recommendations. All new constructs were sequenced to assure Taq polymerase fidelity.

**Cell Culture and Transfections**—COS 7 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Experiments involving transient transfections were carried out as described previously (7) with a total of 2 µg of the appropriate combinations of eukaryotic expression plasmid using Lipofectamine (Life Technologies, Inc.) according to the manufacturer's protocol.

Primary embryonic cortical neuron culture was established from dissected cortices obtained from Sprague-Dawley rat fetuses at embryonic day 17. Cortical cells were dissociated in Neurobasal Medium containing B-27, GutaMax-I and AlbuMax II (Life Technologies, Inc.) and 2 × 10<sup>5</sup> cells/well of a 24-well plate were seeded on glass coverslips precoated with 100 µg/ml poly-D-lysine. Cultures were incubated for 2 weeks at 37 °C in humidified 95% air, 5% CO<sub>2</sub>. Half of the medium was replaced twice weekly. Cultures were characterized by immunocytochemistry using neuron specific antibodies against neurofilament protein (SMI31, Steinberger Meyer Immunochemicals) and astrocyte-specific antibody, glial fibrillary acidic protein (Sigma). Cultures typically were composed of 95% neurons and 5% astrocytes as described previously (22).

**Immunoprecipitations and Immunoblotting**—Immunoprecipitations were performed as described elsewhere (7) using the indicated antibodies. For immunoblotting, immunoprecipitates or 30 µl of cell lysate were separated under reducing conditions by SDS-polyacrylamide gel electrophoresis and immunoblotted as described previously (7, 16).

**Expression and Purification of GST Fusion Proteins**—With the exception of GST-MKK7 and GST-MKK7(KA), GST fusion proteins were expressed in *Escherichia coli* and were prepared, purified, analyzed, and quantified as described previously (5). Purified recombinant GST-MKK4, GST-MKK4(KA), and GST-p46<sup>SAPK</sup> were obtained commercially (Upstate Biotechnology, Inc.). Recombinant GST-MKK7 and GST-MKK7(KA) were prepared as described by Frangioni *et al.* (23). Briefly, following expression in culture, bacterial pellets were washed in 50 ml of ice-cold STE (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) and resuspended in STE containing 100 µg of lysozyme. After a 15-min incubation on ice, dithiothreitol (5 mM final concentration) and Sarkosyl (1.5% final concentration) were added, and the resultant suspension was pulled several times through a 18-gauge needle. Supernatant obtained following centrifugation of the suspension at 10,000 × g for 5 min was adjusted to contain 4% Triton X-100 and purified on glutathione-Sepharose beads. Fusion proteins were eluted from beads with 10 mM reduced glutathione in a buffer containing 75 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, 0.1% Triton X-100 and were concentrated by ultrafiltration using Centricon-10 (Amicon, Inc.).

**In Vitro Kinase Assays**—Cells were transfected as indicated in figure legends. After 24–48 h, cell lysates were prepared as described previously (7) in 1 ml of a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 20 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, and protease inhibitors. Protease inhibitors included 2 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml pepstatin, 0.5 µg/ml leupeptin, and 1 µg/ml aprotinin. p46<sup>SAPK</sup> immune-complex kinase assays were performed as described previously (7). Complexes were incubated for 30 min at 30 °C in 30 µl of kinase buffer (25 mM HEPES, pH 7.2, 10% glycerol, 100 mM NaCl, 20 mM MgCl<sub>2</sub>, 0.1 mM sodium vanadate, and protease inhibitors) containing 25 µM ATP, 5 µCi of [γ-<sup>32</sup>P]ATP (3000 Ci/mmol, Amersham) and 2 µg of GST-c-Jun(1-79). Reactions were terminated by addition of Laemmli buffer, boiled, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and autoradiographed. Where indicated, incorporated counts were counted with a Bio-Rad phosphorimager. Equivalent expression of transfected p46<sup>SAPK</sup> was assessed by immunoblotting. DLK and MLK3 immune-complex kinase assays were performed as above except that 2 µg of GST-MKK4, GST-MKK1, GST-MKK3, GST-MKK5, GST-MKK7, or their kinase negative mutants were substituted as substrate were indicated in figure legends. MKK7 immune-complex kinase assays were carried out as above except that 2 µg of GST-SAPK was substituted as substrate.

**In Vitro SAPK Activation Assays**—*In vitro* activation of SAPK was determined essentially as described (7). FLAG epitope-tagged DLK, DLK(K185A), MLK3, or MLK3(K144E) were immunoprecipitated from 500 µg of transiently transfected COS 7 cell lysate. *In vitro* reconstituted coupled kinase assays were performed by combining indicated

MLK immune complexes with either recombinant GST-MKK4 or GST-MKK7 or their kinase negative mutants as indicated in kinase buffer containing 50  $\mu$ M ATP and 5  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) and incubated at 30  $^{\circ}$ C for 60 min. Recombinant GST-SAPK and GST-c-Jun (1-79)-Sephacryl beads were added, and reaction mixes were incubated for an additional 15 min at 30  $^{\circ}$ C. Reactions were terminated by addition of Laemmli buffer, boiled, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and autoradiographed.

**Affinity Precipitation**—FLAG-DLK, FLAG-MLK3, or vector only were expressed and labeled with [ $^{35}$ S]methionine in a single reaction reticulocyte lysate-based *in vitro* transcription/translation system according to the directions of the manufacturer (Promega). One-tenth of each reaction mix was incubated for 30 min at room temperature with or without 10  $\mu$ g of GST-MKK7 and 25  $\mu$ l of glutathione-agarose in a PBST buffer containing phosphate-buffered 150 mM NaCl (pH 7.4), 1% Triton X-100, and protease inhibitors. Beads were washed five times with PBST then eluted in 5 mM reduced glutathione. Eluates were separated on SDS-PAGE under reducing conditions. Gels were fixed, treated with ENHANCE (DuPont), dried, and autoradiographed.

**Immunocytochemistry**—Adult Sprague-Dawley rats were perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline. Nervous tissues were removed, washed in cold PBS, and cryoprotected in sucrose prior to freezing in liquid nitrogen. Spinal cords were cut in 6- $\mu$ m sections on a cryostat; 40- $\mu$ m sections were obtained with a sliding microtome of brain and cerebellum. Free-floating sections of brain and cerebellum were used for immunocytochemistry as described (24). Primary antibody was detected with species-appropriate horseradish peroxidase-conjugated anti-IgG secondary antibodies and developed with nickel enhanced 3,3'-diaminobenzidine tetrahydrochloride using the Vector Elite detection kit (Vector Laboratories). Indirect immunofluorescence studies utilized complementary secondary antibodies conjugated to Cy2 or Cy3 (The Jackson Laboratory) as indicated in the legends to Figs. 6–9. In studies of primary cortical neurons in culture, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. After exposure to the primary antibody at 4  $^{\circ}$ C overnight, the bound antibody was detected using a secondary antibody conjugated to Cy3 or Cy2.

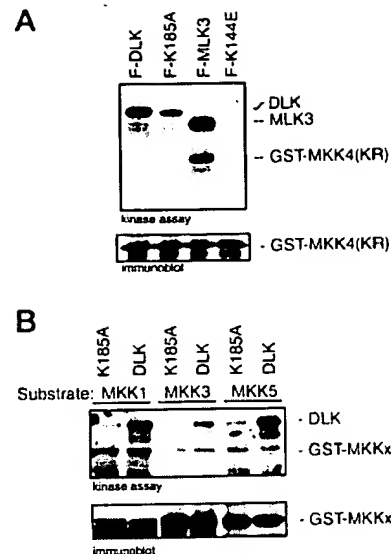
**Subcellular Fractionation Studies**—The preparation of intact nuclei from adult rat cortex and from primary cortical neurons was carried out by isopycnic banding at the 30–35% Iodixanol interface, in order to cause the least possible disruption of the nucleoprotein complexes within them, following the manufacturers protocol (OptiPrep).

**Rat Model of Sciatic Nerve Injury**—Adult male Sprague-Dawley rats weighing 175–225 g were anesthetized with chloral hydrate. The sciatic nerve was exposed in the gluteal region and cut. Control rats were anesthetized and the sciatic nerve was exposed but not cut. At 5 min, 30 min, and 1 day after axotomy, animals were perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline. The spinal cord in the region of the lumbar bulge was removed, washed in cold PBS, cryoprotected in sucrose, and frozen in liquid nitrogen. Three animals at each time point were employed. A minimum of three sections per spinal cord were analyzed by immunofluorescence.

## RESULTS

**Distinct from MLK3, DLK Neither Phosphorylates Nor Activates MKK4 *In Vitro***—Preliminary experiments demonstrated that catalytically inactive MKK4 attenuated the ability of DLK to activate p46<sup>SAPK</sup> when overexpressed in co-transfected COS 7 cells (data not shown and Ref. 6). To more closely examine the potential epistatic relationship between DLK and MKK4, the ability of DLK to phosphorylate MKK4 *in vitro* was investigated. Immunoprecipitated overexpressed DLK, MLK3, or their inactive mutants were incubated with catalytically inactive recombinant GST-MKK4-KR in a buffer containing radio-labeled ATP and magnesium. As reported by others (10, 12), MLK3, but not its inactive mutant, phosphorylated GST-MKK4-KR *in vitro* (Fig. 1A). Contrary to expectation, DLK did not phosphorylate GST-MKK4-KR. In similar experiments, the capacity of immunoprecipitated DLK to phosphorylate *in vitro* catalytically inactive mutants of GST fusion proteins of MKK1, MKK3, or MKK5 was also tested (Fig. 1B). DLK did not phosphorylate recombinant MKK1, MKK3, or MKK5 *in vitro*.

These unexpected results were confirmed by studying the

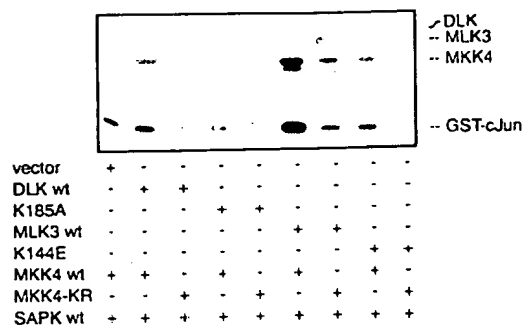


**FIG. 1. MLK3 but not DLK phosphorylates recombinant MKK4 *in vitro*.** A, FLAG-tagged DLK (F-DLK), MLK3 (F-MLK3), or catalytically inactive DLK(K185A) (F-K185A) or MLK3(K144E) (F-K144E) were immunoprecipitated from lysates prepared from transiently transfected COS 7 cells. Indicated immunoprecipitates were assayed *in vitro* for their capacity to phosphorylate purified recombinant GST-MKK4(KR). Reaction mixes were separated on 12.5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. Immunoblots from corresponding experiments were used to evaluate relative abundance of GST-MKK4(KR) in each reaction. B, FLAG-tagged DLK (F-DLK) or DLK(K185A) (F-K185A) were immunoprecipitated from lysates prepared from transiently transfected COS 7 cells. Indicated immunoprecipitates were assayed *in vitro* for their capacity to phosphorylate bacterially expressed and purified recombinant kinase-negative GST-MKK1, GST-MKK3, or GST-MKK5 as indicated. Immunoblots from corresponding experiments were used to evaluate relative abundance of indicated GST-MKK in each reaction. Experiments were repeated three times with similar results.

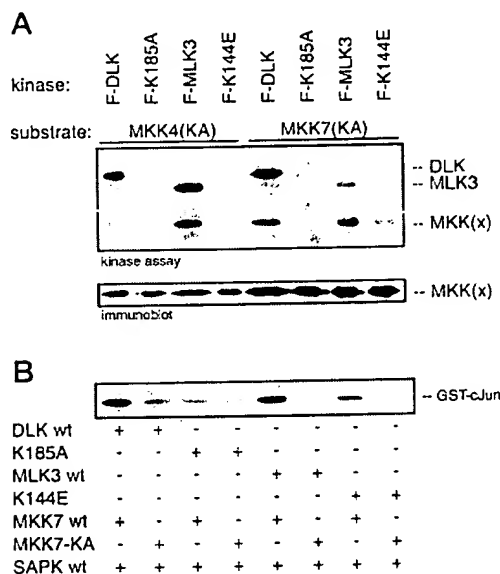
ability of immunoprecipitated DLK to activate MKK4 in an *in vitro* reconstitution experiment. Immunoprecipitated overexpressed DLK, MLK3, or their catalytically inactive mutants were sequentially combined in the presence of ATP and magnesium with recombinant MKK4 (or its kinase negative mutant) and SAPK. SAPK activation was assayed by capture of SAPK onto GST-c-Jun-agarose beads followed by kinase assay in the presence of [ $\gamma$ - $^{32}$ P]ATP. As reported previously, MLK3 but not its kinase negative mutant activated MKK4 and ultimately SAPK (Fig. 2) (10, 12). However, in three repeated experiments, DLK did not activate MKK4 *in vitro*.

**DLK Phosphorylates and Activates MKK7 *In Vitro***—By analysis of primary structure, MLK2 and MLK3 form a closely related group structurally distant from DLK. Therefore, it was hypothesized that DLK utilizes substrate or substrates distinct from MKK4. The recently identified MAP kinase kinase MKK7/JNKK2 shown to specifically activate SAPK was considered a potential candidate substrate (15, 25, 26). To test this possibility, *in vitro* kinase assays were repeated as above. Indeed, immunoprecipitated DLK phosphorylated a recombinant catalytically inactive GST-MKK7 fusion protein *in vitro*; immunoprecipitated MLK3 behaved in a similar fashion (Fig. 3A). In control experiments, neither immunoprecipitated catalytically inactive DLK (K185A) nor catalytically inactive MLK3 (K144E) phosphorylated GST-MKK7-KA.

*In vitro* reconstitution coupled kinase assays were used to confirm and extend these observations. As reported previously by others (15, 26), bacterially expressed and purified wild type GST-MKK7 activated rSAPK *in vitro* without known prior activation by a MAPKKK. To confirm that MKK7 catalytic

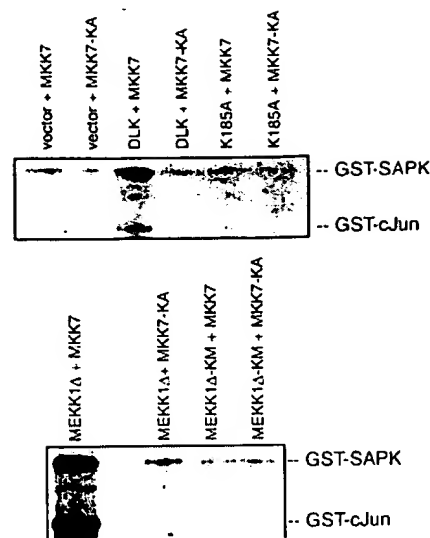


**FIG. 2. MLK3 but not DLK activates MKK4 *in vitro*.** FLAG-tagged DLK (DLK wt), MLK3 (MLK3 wt), or catalytically inactive DLK(K185A) (K185A) or MLK3(K144E) (K144E) were immunoprecipitated from lysates prepared from transiently transfected COS 7 cells. Immunoprecipitates were combined as indicated with purified bacterially expressed GST-MKK4 (MKK4 wt) and GST-p46<sup>SAPK</sup> (SAPK wt), or catalytically inactive MKK4 (MKK4-KR), and incubated at 30 °C for 20 min in the presence of ATP. Activation of SAPK was assayed in an *in vitro* kinase assay following incubation of reaction mixes with GST-c-Jun-Sepharose beads as described under "Materials and Methods." Experiments were repeated three times with similar results.



**FIG. 3. DLK and MLK3 phosphorylate and activate MKK7 *in vitro*.** **A**, FLAG-tagged DLK (F-DLK), MLK3 (F-MLK3), or catalytically inactive DLK(K185A) (F-K185A) or MLK3(K144E) (F-K144E) were immunoprecipitated from lysates prepared from transiently transfected COS 7 cells. Indicated immunoprecipitates were assayed *in vitro* for their capacity to phosphorylate bacterially expressed and purified recombinant GST-MKK7-KA or GST-MKK4-KA. Reaction mixes were separated on 12.5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. Immunoblots from corresponding experiments were used to evaluate relative abundance of GST-MKK7(KA) or GST-MKK4(KA) in each reaction. **B**, FLAG-tagged DLK (F-DLK wt), MLK3 (F-MLK3 wt), DLK(K185A) (F-K185A), or MLK3(K144E) (F-K144E) were immunoprecipitated from lysates prepared from transiently transfected COS 7 cells. Immunoprecipitates were combined as indicated with purified GST-MKK7 (MKK7 wt) or catalytically inactive GST-MKK7 (MKK7-KA) and incubated at 30 °C for 60 min in the presence of ATP. Samples were incubated with recombinant GST-p46<sup>SAPK</sup> (SAPK wt) and GST-c-Jun and activation of SAPK was assayed in an *in vitro* kinase assay. Experiments were repeated three times with similar results.

activity was required for SAPK activation in these experiments, a mutant GST-MKK7 was created in which the invariant lysine in subdomain II (Lys<sup>76</sup>, known to stabilize ATP in the ATP binding site in other kinases) was replaced with alanine. GST-MKK7(KA) mutant failed to activate rSAPK *in vitro*. Given these results, we examined whether overexpressed and



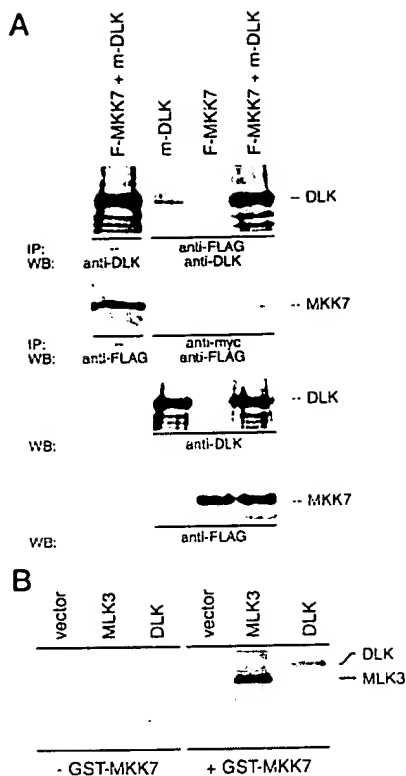
**FIG. 4. Overexpressed DLK activates MKK7 *in vivo*.** COS 7 cells were co-transfected as indicated with plasmids encoding FLAG-tagged MKK7 or catalytically inactive FLAG-tagged MKK7-KA and with plasmids encoding vector only, Myc-tagged DLK, MEKK1Δ, or catalytically inactive myc-DLK(K185A) or MEKK1Δ-KM. Cells were lysed after 24 h and immunoprecipitated FLAG-MKK7 was combined with recombinant GST-SAPK and GST-c-Jun and assayed for catalytic activity *in vitro* in a coupled kinase assay.

immunoprecipitated DLK or MLK3 would potentiate the activation of GST-MKK7 *in vitro*. Immunoprecipitated DLK and MLK3, but not their catalytically inactive mutants, further activated recombinant GST-MKK7 and ultimately SAPK (Fig. 3B). Substitution of the mutant GST-MKK7-KA in reconstitution experiments blocked the ability of immunoprecipitated DLK and MLK3 to activate SAPK.

**DLK Activates MKK7 *In Vivo***—Experiments were performed to confirm that DLK activates MKK7 *in vivo*. COS 7 cells were transiently co-transfected with plasmid encoding FLAG-tagged MKK7 and either Myc-tagged DLK or catalytically inactive Myc-DLK (K185A). Activation of immunoprecipitated MKK7 was evaluated in an *in vitro* coupled kinase assay using recombinant SAPK and GST-c-Jun (1–79) as substrate (Fig. 4). Expression of DLK but not catalytically inactive K185A resulted in the activation of MKK7. In control experiments, when DLK was co-expressed with catalytically inactive MKK7-KA, immunoprecipitated MKK7-KA failed to phosphorylate rSAPK *in vitro*. These results demonstrate that overexpressed DLK can activate overexpressed MKK7 *in vivo*.

**DLK Associates with MKK7 *In Vivo* and *In Vitro***—To examine whether overexpressed DLK interacts *in vivo* with overexpressed MKK7, myc-tagged DLK was co-expressed with FLAG-tagged MKK7 in COS 7 cells. As shown, FLAG-tagged MKK7 co-immunoprecipitated with myc-tagged DLK (Fig. 5A). To confirm a direct interaction between DLK and MKK7, metabolically labeled DLK expressed in a reticulocyte lysate system was incubated with bacterially expressed and purified GST-MKK7 or control. As shown, DLK co-purified only in the presence of GST-MKK7 when isolated by glutathione-agarose affinity chromatography (Fig. 5B). MLK3 behaved in a similar fashion. Taken together with the results of the experiments described above, these results provide evidence that MKK7 may serve as a necessary intermediate in a pathway between DLK or MLK3 and SAPK activation *in vivo*.

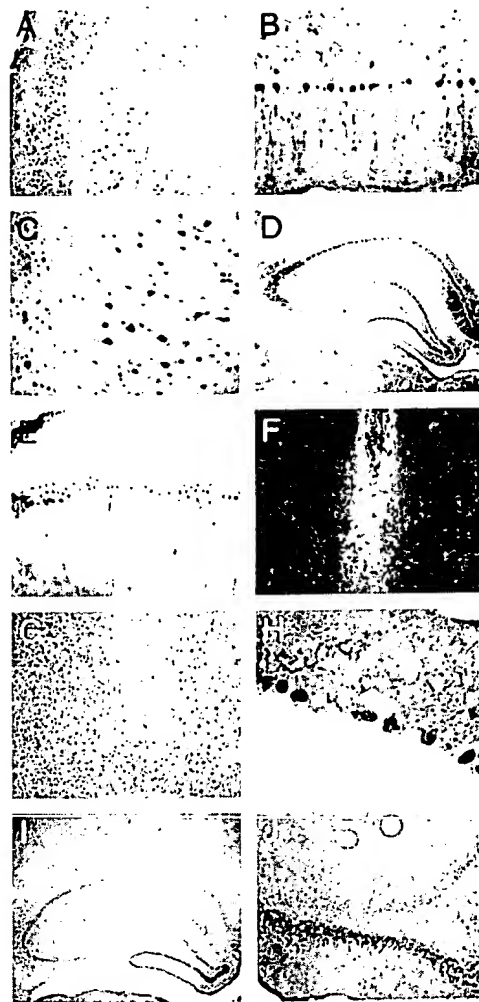
**Distribution of DLK, MLK3, and MKK7 Proteins in Normal Adult Rat Nervous System**—To obtain evidence supporting the epistatic relationship between endogenous DLK and MLK3 and their potential substrates in brain, the subcellular localization



**Fig. 5. DLK associates with MKK7.** *A*, COS 7 cells were cotransfected with indicated combinations of plasmid encoding Myc-tagged DLK (0.5  $\mu$ g), FLAG-tagged MKK7 (0.5  $\mu$ g), FLAG-MKK7-KA (0.5  $\mu$ g), or empty vector (to 2  $\mu$ g total). Cell lysates were immunoprecipitated as indicated with anti-Myc (9E10) or anti-FLAG (M2) antibody. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, then immunoblotted as indicated with anti-DLK polyclonal serum (C1) or anti-FLAG (M2) antibody. Twenty  $\mu$ g of whole cell lysate obtained from Myc-DLK and FLAG-MKK7 transfected cells were run in the left lanes and after immunoblotting with the indicated antibodies served as mobility markers. *B*, DLK and MLK3 or vector-only control were *in vitro* transcribed and translated in the presence of [ $^{35}$ S]methionine. Samples were either mixed or not mixed with GST-MKK7 as indicated. Following incubation with glutathione-agarose, samples were washed and eluted with reduced glutathione. Eluates were separated by SDS-PAGE and visualized by autoradiography.

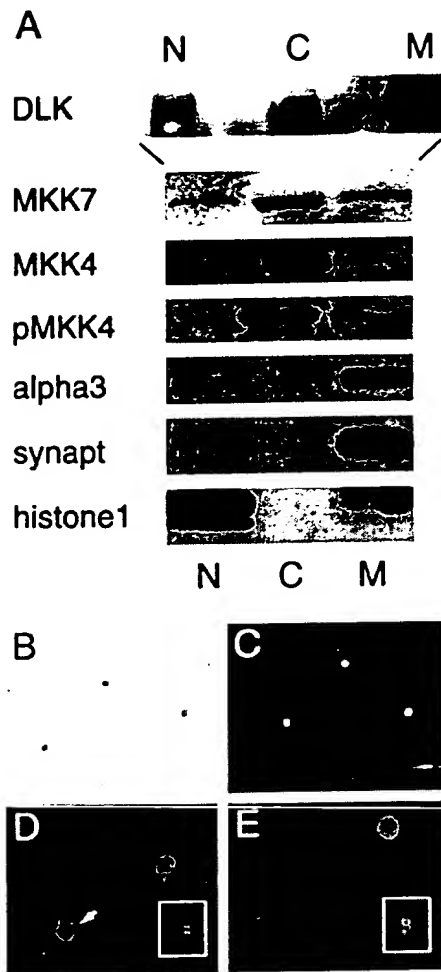
of DLK, MLK3, and MKK7 proteins was examined by immunohistochemistry (Fig. 6). Using the previously characterized C1 polyclonal serum generated against a COOH-terminal DLK-GST fusion protein (5), DLK immunoreactivity was detected in neurons of the central nervous system (Fig. 6, *A–D*). This finding was consistent with previous observations using *in situ* RNA hybridization in which DLK transcript was widely detected in neurons of the central and peripheral nervous system (16). In pyramidal cells of the cerebral cortex and in Purkinje cells of the cerebellum, strong DLK immunoreactivity was also detected in apical dendrites. MLK3 immunoreactivity could not be distinguished above background in neurons. Instead, MLK3 protein was identified primarily in cells of the periventricular ependyma, choroid plexus, and meninges. (Fig. 6, *E* and *F* not shown). Using a previously characterized MKK7 rabbit antiserum (24), MKK7, like DLK, was identified predominantly in neurons of the central nervous system (Fig. 6, *G–J*). Whereas DLK and MKK7 immunoreactivity was identified in the same cells of the cerebral cortex and cerebellum, regional differences were detected in the intensity of MKK7 and DLK staining (compare granule cells of the dentate gyrus of hippocampus, Fig. 6, *D*, *I*, and *J*).

**Identification of DLK, MKK7, and MKK4 in Cellular Subfractions**—Several experiments were undertaken to study the



**Fig. 6. Indirect immunohistochemistry demonstrating localization of DLK, MLK3, and MKK7 in normal adult rat nervous system.** DLK (*A–D*) and MKK7 protein (*G–J*) co-localized within neurons of the central and peripheral nervous system including those of the cerebral cortex (compare *A* and *G*) and cerebellum (*B*, *H*). MLK3 protein was identified in the ependyma (*E*, *F*) but could not be identified in neurons even by immunofluorescence (*F*). Note DLK and MKK7 immunoreactivity within dendritic processes and accentuated staining in a perinuclear and nuclear distribution (*C*, *H*). Within the neurons of the hippocampus, MKK7 immunoreactivity was most prominent within the neurons of the lower leaflet of the dentate gyrus (*I*, *J*), whereas DLK staining was more uniformly distributed (compare *D* and *I*, *J*). Purkinje cells of the cerebellum and their apical dendrites stained intensely for DLK and MKK7 (*B*, *H*). Note differences in staining intensity between DLK and MKK7 within the granular cell layer (*B*, *H*).

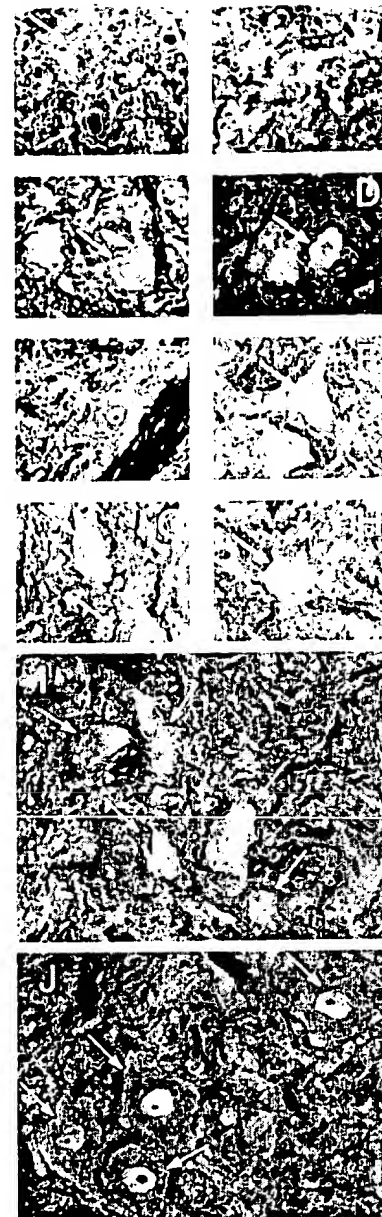
subcellular localization of DLK, MKK7, and MKK4. Normal rat cerebral cortex was disrupted and cells were subfractionated by isopycnic banding. Nuclear, cytosolic, and microsomal fractions were identified by immunoblotting using subfraction specific markers (Fig. 7*A*). The purity of isolated nuclear preparations were demonstrated as shown in Fig. 7, *B* and *C*. Following separation of subfraction lysates on SDS-PAGE and immunoblotting, DLK was identified most abundantly in the microsomal fraction but was also prominent in the cytosol. Like MKK4, MKK7 was particularly enriched in the cytosolic fraction. A small but detectable quantity of DLK and MKK7 were identified within the nuclear fraction (Fig. 7*A*). Neither antibodies directed against phosphorylated-MKK4 (New England Biolabs) nor phosphorylation-independent antibodies directed against MKK4 (Sigma and Santa Cruz) recognized MKK4 in the nuclear fraction.



**FIG. 7. Localization of DLK, MKK7, and MKK4 in subfractionated cerebral cortex.** A, cerebral cortex was homogenized and cells were subfractionated as described under "Materials and Methods." Twenty- $\mu$ g aliquots of nuclear (N), microsomal (M), and cytosolic (C) fractions were separated on SDS-PAGE, transferred to nitrocellulose, then immunoblotted as indicated with antibodies directed against DLK, MKK7, MKK4, and phosphorylated MKK4. Fractions were also immunoblotted with antibodies specific for  $\alpha_3$  subunit of sodium-potassium ATPase ( $\alpha_3$ ), synaptophysin (*synapt*), and histone 1 to demonstrate suitability of fractionation. In order to demonstrate the purity of the nuclear preparation, an aliquot was stained with DAPI indicating the presence of DNA. Shown is an identical field visualized both by phase (B) and immunofluorescence microscopy (C). Immunofluorescence and confocal microscopy were used to analyze additional aliquots for the presence of DLK (D) and MKK7 (E). Insets represent reconstructions of confocal images in the z plane. Control experiments lacking primary antibody or using primary antibody preadsorbed with a molar excess of appropriate fusion protein failed to stain isolated nuclei (data not shown).

To assure that the nuclear localization of DLK and MKK7 suggested by the immunoblotting experiments above was not due to contamination of nuclear fractions with associated microsomal membranes, isolated nuclei from rat cerebral cortex were fixed and analyzed by indirect immunofluorescence. Confocal microscopy demonstrated homogeneous distribution of DLK and MKK7 protein throughout the nucleus (Fig. 7, D, E, and insets).

**DLK Translocates to the Nucleus in an *in Vivo* Model of Neuronal Injury**—Several components of the various MAP kinase pathways have been demonstrated to translocate within cells following appropriate stimulation. For this reason, the subcellular localization of DLK was also examined by immunofluorescence microscopy in an *in vivo* model of neuronal injury.



**FIG. 8. DLK translocates to the nucleus following truncation of the sciatic nerve in an *in vivo* model of cellular injury.** At indicated times following sciatic nerve axotomy, normal adult rats were perfused fixed with 4% paraformaldehyde by left ventricular puncture. Spinal cord in the region of the lumbar bulge was removed, prepared, and studied by immunofluorescence microscopy. Shown are representative sections of immunofluorescence staining of motor neurons for DLK (A, C, E, G, I, J) and MKK7 (B, D, F, H) in control animals (A, B, I) and at 5 min (C, D), 30 min (E, F), and 24 h (G, H) following axotomy. Arrows emphasize location of nuclei. Note DLK immunoreactivity along plasma membranes best visualized in A, E, and J. I and J show a larger field of lumbar spinal cord sections 20 min after sham operation or sciatic nerve axotomy, respectively.

Axotomy of the rat sciatic nerve led to translocation of DLK from a predominantly non-nuclear cell compartment to a distinctly nuclear localization within motor neurons of the lumbar spinal cord beginning within 5 min of axonal injury (Fig. 8). Strong nuclear DLK immunoreactivity was maintained at 30 min and at 24 h following injury. Three animals at each time point were examined and a minimum of three sections per spinal cord was evaluated by indirect immunofluorescence for each animal studied. Sham-operated animals were used as controls at each time point studied. In these control animals,



the intensity of DLK immunoreactivity in the nucleus was never observed to be above that observed in the cytoplasm. However, in sections from injured animals, from 2 to 14 cells per section exhibited dramatically increased nuclear localization of DLK. Phosphorylated MKK4 was not identified in the cell nucleus either in control animals or following axotomy at any time point studied (data not shown). However, MKK7, like DLK, accumulated within the nucleus following axotomy.

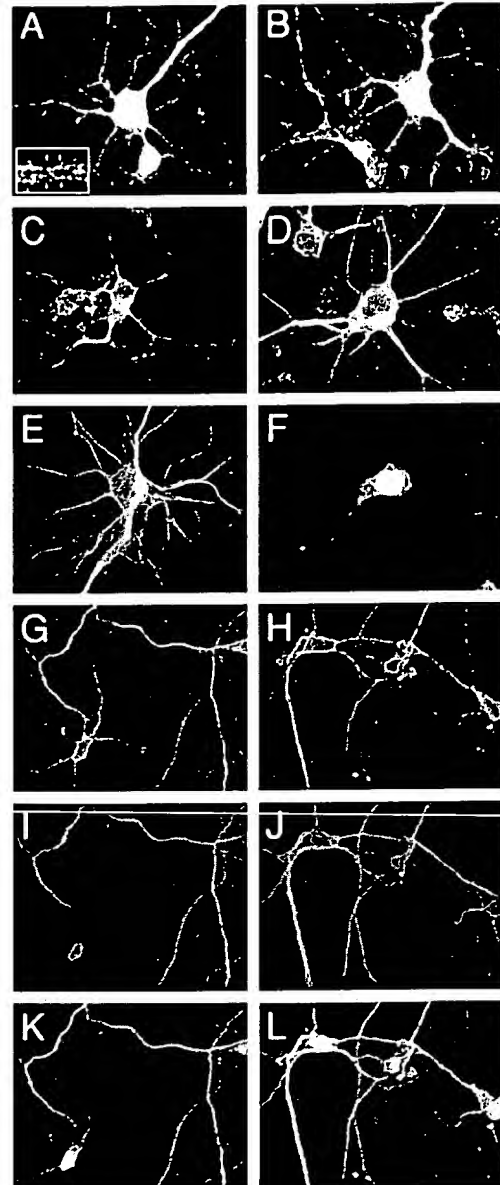
**DLK Immunoreactivity Co-localizes with That of MKK7 in Neuronal Culture**—To investigate the subcellular localization of DLK, MKK7, and MKK4 proteins in an additional system, embryonic rat cortical neurons in primary culture were examined by immunocytochemistry. DLK immunoreactivity was detected in a prominent perinuclear and intranuclear distribution in neurons (Fig. 9, A, G, I, and K). DLK was also detected within all neuronal processes and was prominently associated with plasma membrane (Fig. 9, A, G, I, K, and Fig. 9A, inset). This was consistent with our published observations that DLK was associated with plasma membrane fractions isolated from synaptosomal preparations (16). Like DLK, MKK7 immunoreactivity was identified in perinuclear and intranuclear compartments as well as in cell processes (Fig. 9, B, H, J, L). Using three distinct commercially obtained antibodies, MKK4 immunoreactivity was detected in neuron cell processes and in a non-nuclear distribution within the neuron cell body that was distinct from that of DLK and MKK7 (Fig. 9, C and D).

The observations that DLK and MKK7 occupied compartments distinct from MKK4 suggested the hypothesis that spatially and functionally distinct SAPK pathways composed of unique components exist within individual cells. To examine this hypothesis further, the subcellular distribution of SAPK $\beta$ /JNK3 and SAPK $\gamma$ /JNK1 were investigated by immunohistochemistry in primary neuronal culture. SAPK $\beta$ /JNK3 immunoreactivity was identified exclusively in the neuronal nuclei (Fig. 9F). In contrast, SAPK $\gamma$ /JNK1 immunoreactivity was extranuclear and localized predominantly to the neuronal processes (Fig. 9E). Therefore, different SAPK species and different MKK species occupy distinct compartments within neurons.

#### DISCUSSION

This paper establishes DLK as a MAPK kinase kinase capable of associating with, phosphorylating, and activating MKK7. That DLK functions in this role in a physiologic setting is supported by observations that endogenous DLK and MKK7 are present in the same compartments within neurons. Moreover, both biochemical evidence and analysis of subcellular localization indicate that DLK does not use MKK4 as an intermediate in SAPK activation.

MAPKKK substrate specificity appears to be defined by the combination of several factors that include (but may not be limited to) the biochemical affinity of the kinase for its substrate, cell type-specific expression, and differential targeting of unique MAPKKKs to specific subcellular compartments. The results presented herein provide an example of each of these determinants. In a fashion reminiscent of the specificity of MKK3 and MKK6 for p38<sup>mapk</sup>s (27–29) and of MKK7 for the p46/p54<sup>SAPK</sup>s (15, 25, 26, 30), DLK appears to possess enzymatic specificity for MKK7. Conversely, our results demonstrate that MKK7 may serve as a substrate for several MAPKKKs, including MLK3 and MEKK1. Precedence from the SAPK pathway literature suggests that this is not an unexpected paradox since MKK4 appears to behave as a substrate for multiple MAPKKKs at least in biochemical assays (reviewed in Ref. 2). However, by imposing a number of additional determinants of interaction specificity, nature appears to have restricted interactions between specific kinases and substrates



**Fig. 9. DLK immunoreactivity co-localizes with that of MKK7 in embryonic cortical neurons in primary culture.** Indirect immunofluorescence microscopy was used to investigate the subcellular localization of DLK (A, G), MKK7 (B, H), MKK4 (C), phosphorylated-MKK4 (D), SAPK $\beta$ /JNK1 (E), and SAPK $\gamma$ /JNK3 (F) in rat cortical neurons in primary culture. Enlargement of neuronal process in A (inset) demonstrates localization of DLK protein along plasma membrane. Cells were double-labeled for neurofilament protein (Cy3, red) (I, J) and for either DLK (G) or MKK7 (H) (both Cy2, green). Double photographic exposures to demonstrate superimposition of staining patterns for DLK and neurofilament protein (K) or MKK7 and neurofilament protein (L) are shown.

in the SAPK-like pathways.

That DLK and MLK3 are expressed in different cell types in the nervous system provides one example by which interactions between members of the MLK family and specific MAPK kinases may be restricted. Specification of subcellular compartmentation within individual cells may provide a second mechanism determining these interactions. Recognition that various MAPK kinases and MAPK kinase kinases are targeted to distinct subcellular compartments supports this hypothesis. The subcellular localization of endogenous DLK within neurons is complex. In the nerve terminal, endogenous DLK is present both in cytosol and in subcellular fractions enriched in plasma

membrane (16). In the cell body, DLK occupies compartments that include the plasma membrane, cytosol, and nucleus. Recently, the distinct subcellular compartmentation of other MAPKKs has also been described. Endogenous MEKK family members occupy distinct subcellular compartments in COS cells including Golgi, post-Golgi, and nuclear compartments (31), and the mixed lineage kinase MLK2 co-localizes with phosphorylated JNK1/2 along microtubules in injected Swiss 3T3 cells (9). Our results show that like the MAPKKs, endogenous MKK4 and MKK7 also occupy distinct neuronal subcellular compartments.

Our intent in examining the subcellular localization of DLK and its potential MAPK kinase substrates was to provide evidence supporting the biochemical data suggesting that endogenous DLK uses MKK7 and not MKK4 as substrate. Consistent with the biochemical data, the subcellular compartmentation of DLK and MKK7 are similar in neurons; the subcellular compartmentation of MKK4 is distinct from that of DLK and MKK7. Because DLK and MKK7 have overlapping but not identical subcellular compartmentation in neurons, genetic and other experimental approaches will be necessary to further validate the hypothesis that endogenous DLK specifically uses MKK7 as substrate.

SAPK isoforms also appear to be differentially compartmentalized within neurons. Ten splice isoforms derived from three SAPK/JNK genes have been isolated from brain (32). Although in most cases the specific splice isoform has not been studied, SAPK species have been identified associated with several cellular compartments, including cytosol, microtubules (9), and nuclei (13, 33–36). Our observation that endogenous SAPK $\gamma$ /JNK1 and SAPK $\beta$ /JNK3 occupy distinct subcellular compartments in the same cell extends these observations. Beyond differences in subcellular compartmentation, limited evidence presently exists to support the hypothesis that discretely localized SAPK species within the same cell have dissociated function. The most direct evidence presently available in this regard derives from the observation that genetic deletion of JNK3, but not deletion of JNK1 or 2, protects mice from hippocampal neuron injury and apoptotic cell death associated with chronic administration of a glutamate receptor-agonist (37). That SAPK $\beta$ /JNK3 and SAPK $\gamma$ /JNK1 are differentially compartmentalized within individual neurons is consistent with the results of these JNK null-mutant experiments. Together, these observations suggest the existence of spatially and functionally discrete SAPK species within the same cell.

Translocation between subcellular compartments may provide an additional mechanism by which MLK protein kinases gain access to their specific substrates. This report documents that DLK and MKK7 can translocate to the cell nucleus. The mechanism and functional significance of translocation of DLK and MKK7 to the neuronal nucleus following axonal injury will require additional detailed study. Each of these protein kinases is present in neuronal nuclei *in vivo* under basal conditions, yet each accumulates following axotomy. That nuclear accumulation of DLK occurs rapidly suggests that this kinase translocates from extranuclear pools of pre-existing protein. The nuclear translocation of MAP kinase kinases following a cellular stimulus has not been observed previously, although MEKK1 has been identified in the nucleus of COS cells under basal conditions (7). It is not known whether stimulus-induced nuclear translocation is a response common to other MAPKKK proteins.

Activation of SAPK (particularly SAPK $\beta$ /JNK3) has been implicated in the neuronal response to various types of cellular injury (19, 21, 35). SAPK-activating and -interfering mutant overexpression studies performed in PC12 cells first implicated

SAPK in the neuronal apoptotic response to nerve growth factor withdrawal (34). Recent studies demonstrated that SAPK $\beta$ /JNK3 translocates to the nucleus of hippocampal neurons following hypoxic injury in humans (37). Indeed, deletion of SAPK $\beta$ /JNK3 protects mice from hippocampal neuron injury and programmed cell death associated with chronic administration of a glutamate receptor agonist (35). Finally, it was recently reported that sciatic nerve axotomy induces sustained SAPK, c-Jun, and AP-1 activation in lumbar dorsal root ganglion axons (19). Rapid and sustained translocation of DLK corresponds temporally with this activation. Combined with these observations, the results reported herein provide provocative preliminary evidence suggesting that DLK participates in a cellular response to axonal injury mediated by MKK7 and SAPK $\beta$ /JNK3.

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